Dedicated to
Giuliano and Luciano,
reason of my life

UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

Dottorato di Ricerca in Genetica e Medicina Molecolare

Tesi Sperimentale di Dottorato

"Hypermutation: molecular and phenotypic aspects of Mismatch Repair and RecBCD recombination altered pathways in *Neisseria meningitidis* virulent strains"

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Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano"

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Neisseria meningitidis: HISTORY AND INTRODUCTION

The meningococcus and meningococcal disease have been of interest to scientists for more than 100 years. To depress the frequency of mortality from meningococcal disease, a deeper understanding of the physiology and adaptability of the meningococcus and of the mechanisms of microbial pathogenicity is necessary.

Meningococcal disease was first described as “epidemic cerebrospinal fever” by Vieusseaux in 1805 (Vieusseaux, 1805). However, it was not until 1887 that Anton Weichselbaum in Vienna isolated the meningococcus from cerebrospinal fluid (Weichselbaum, 1887). In 1898 Councilman and co-workers firmly established the etiologic agent of epidemic cerebrospinal meningitis when they found meningococcus in the cerebrospinal fluid of 31 of 34 cases (Councilman et al., 1898).

Although much has been written about this gram-negative pathogen and the variety of clinical manifestations of meningococcal disease, little is known of the direct relationship between the physiology and the biochemistry of the microbe, on the one hand, and the pathogenesis of disease on the other.

The vast literature on meningococci stems from a curiosity about this bacterium, which harmlessly inhabits the oropharynx of a portion of the normal population without overt symptoms of disease, yet is one of the fastest killers of humans among biological agents. Paradoxically, the meningococcus can cause low-frequency sporadic disease in the population or large and fast-spreading epidemics. Moreover, the clinical manifestation of meningococcal disease may vary greatly as well, from those associated with chronic meningococcemia at the one extreme (Sanders et al., 1968) to those of fulminant meningococcal disease with associated coagulopathy on the other. The pathological picture also varies widely, depending on the

severity of the disease, from relatively insignificant to the involvement of all major organs including the brain.

Neisseria meningitidis is gram-negative and can appear as a single coccus or diplococcus. The flattened adjacent sides give the organism the typical "biscuit" or "kidney bean" shape when a fresh specimen is examined (Fig. 1).

Figure 1. Electron micrograph of *Neisseria meningitidis*

It is important that laboratory processing be done rapidly because autolysis occurs with this organism. Although meningococci are not as nutritionally fastidious as gonococci, specimens from patients with possible meningococcal disease must be cultured on enriched media. If inoculated promptly, meningococci will grow on many different solid media such as blood agar, supplemented chocolate agar, Muller-Hinton agar, or GC-agar (GonoCoccal agar). In addition, Thayer Martin media can be used for oropharyngeal cultures. *Neisseria* species are fastidious in their growth conditions, and all media should be incubated at 35°C to 37°C under

increased carbon dioxide content (3% to 10%) and humidity. The final identification of this organism is confirmed by a characteristic carbohydrate fermentation pattern (dextrose-positive, maltose-positive, and sucrose-negative) and being oxidase-positive or by direct latex agglutination testing.

The meningococcus produces a capsular polysaccharide that is the basis of classifying this organism into 13 different serogroups. The serogroups A, B, C, X, Y, Z, 29-E, W-135, and a newly described serogroup, L, have well-characterized capsular polysaccharides (Ashton et al., 1983). The capsular polysaccharides of serogroups D, H, I, and K have not been fully characterized.

Of these, five serogroups (A, B, C, Y, and W-135) are responsible for most of the disease worldwide (Peltola, 1983). Meningococci can be further subdivided into serotypes and sub-serotypes by the detection of serologically distinct epitopes present on their class 2 or class 3 (PorB) and class 1 (PorA) OMPs (other membrane proteins) (Frash et al., 1985). The population biology of meningococci has been also studied by multilocus enzyme electrophoresis and multilocus sequence typing. Despite the genetic diversity of the organisms, a few electrophoretic types, such as subgroup I, subgroup III, ET-5, ET-37, lineage 3, and cluster A4, are repeatedly identified from invasive disease cases (Caugant, 2001).

The meningococcus may possess the ability to develop pili, which can enhance attachment to pharyngeal cells of the host (DeVoe, 1982). This ability may influence the epidemiology and pathogenicity of this organism.

The importance of a bactericidal antibody and an intact complement system in protecting a host from meningococcal infection is well documented. Goldschneider and associates have shown that in persons with exposure to the organism who lack a serum bactericidal antibody to the infecting strain, there is a 30% to 50% chance of meningococcal disease developing (Goldschneider, et al., 1969). This lytic antibody is directed primarily against the capsular

polysaccharide and forms the basis for the development of potentially effective vaccines. In addition, lytic antibody has been found to be directed against noncapsular antigens, accounting for most of the "natural immunity" to meningococcal disease (Goldschneider, et al., 1969; Reller et al., 1973).

EPIDEMIOLOGY OF THE NEISSERIAL INFECTION

The meningococcus has its natural habitat in the mucous membranes of the oropharynx of normal human hosts. Those harbouring the organism are usually asymptomatic and are commonly referred to as "carriers". The frequency of carriage in the normal population ranges from 5% to 30% (Griffiss & Artenstein, 1976) during nonepidemic periods but may approach 100% during epidemics.

The oropharynx serves as the reservoir for the spread of meningococcal infection within the population; the microorganism is disseminated by means of aerosols (Artenstein, et al., 1968; Glover, 1918; 1920). Airborne organisms impinge on the mucus-covered surfaces of the oropharyngeal cells, where they adhere and establish a population. As human beings are the only natural host for the meningococcus (Griffiss & Artenstein, 1976), it seems reasonable to assume that there is some unique property in the human throat, such as a specific receptor, that allows the colonization of the human oropharyngeal mucosa but not that of animals.

The colonization of the oropharynx by meningococci elicits host antibodies of the three major immunoglobulin classes within 7 to 10 days (Goldschneider, et al., 1969). In many instances such antibodies seem unable to eliminate the microorganism from the oropharynx, since the host may remain a carrier of a specific strain for weeks or even months (Gotschlich et al., 1969).

As approximately 50% of meningococcal isolates taken from the oropharynx appear to be noncapsulated (Frasch & Robbins, 1978), it is possible that such strains can survive prolonged carriage in the presence of serum antibodies. Investigators found that those harbouring gonococci had twice the incidence of meningococcal carriage, suggesting a general susceptibility to the carriage of, or perhaps infection by, pathogenic *Neisseriae* (Ødegaard & Gedde-Dahl, 1979).

During both epidemic and nonepidemic periods, there is a seasonal fluctuation in which the mean monthly incidence is most often at its lowest during the summer and early fall and peaks during February, March, or April in the northern hemisphere (May, 1980). Age-specific attack rates decrease with age. Most cases occur in children younger than 5 years, and slightly more males than females are affected. In addition, overcrowding and poor living conditions predispose persons to meningococcal infection and disease. Contacts of patients with meningococcal infections who sleep and eat in the same household have a significantly increased risk of developing the disease compared with the general population, and they must receive chemoprophylaxis.

The endemic rate of meningococcal disease in the United States over the past 40 years has varied from 1 to 3 per 100,000. Attack rates during epidemics can exceed 100 cases per 100,000 population (CDC: Centers for Disease Control, MMWR, 1981; 1985).

In Italy, the annual incidence of meningococcal disease is of 3-6 cases per 1,000,000 per year. In 2001 there were 203 cases of meningococcal meningitis, in 2002 were reported 225 cases, and 131 cases in 2003 (data Istituto Superiore di Sanità, Roma, Italy).

Meningococcal disease has been the focus of increasing attention recently due to its changing patterns and the apparent rise in its incidence in several parts of the world where it was not considered previously to be a public health problem. Meningococcal disease continues to be a problem in Africa, and at the same time, deaths due to this disease have risen during the past 10 years in South America, the Middle East, Southern Africa, Europe, and Asia (Aymes et al., 1976). Most of these outbreaks around the world are caused by serogroups A and C (Fig. 2). In Italy, since 1999 the proportion of serogroup C *Neisseria meningitidis* organisms has been increasing while the proportion of serogroup B organisms, which have predominated in the past, has decreased. Recently 46% of the organisms were serogroup C.

Before the advent of sulfonamides, the case-fatality ratio for meningococcal disease was 75% to 90 % (Bauks, 1948), but with vigorous antimicrobial treatment the case-fatality ratio can be brought below 15%. At present, antibiotic sensitivity testing indicates that circulating *Neisseria meningitidis* strains are resistant to sulfadiazine but sensitive to rifampin, which is used in chemoprophylaxis.

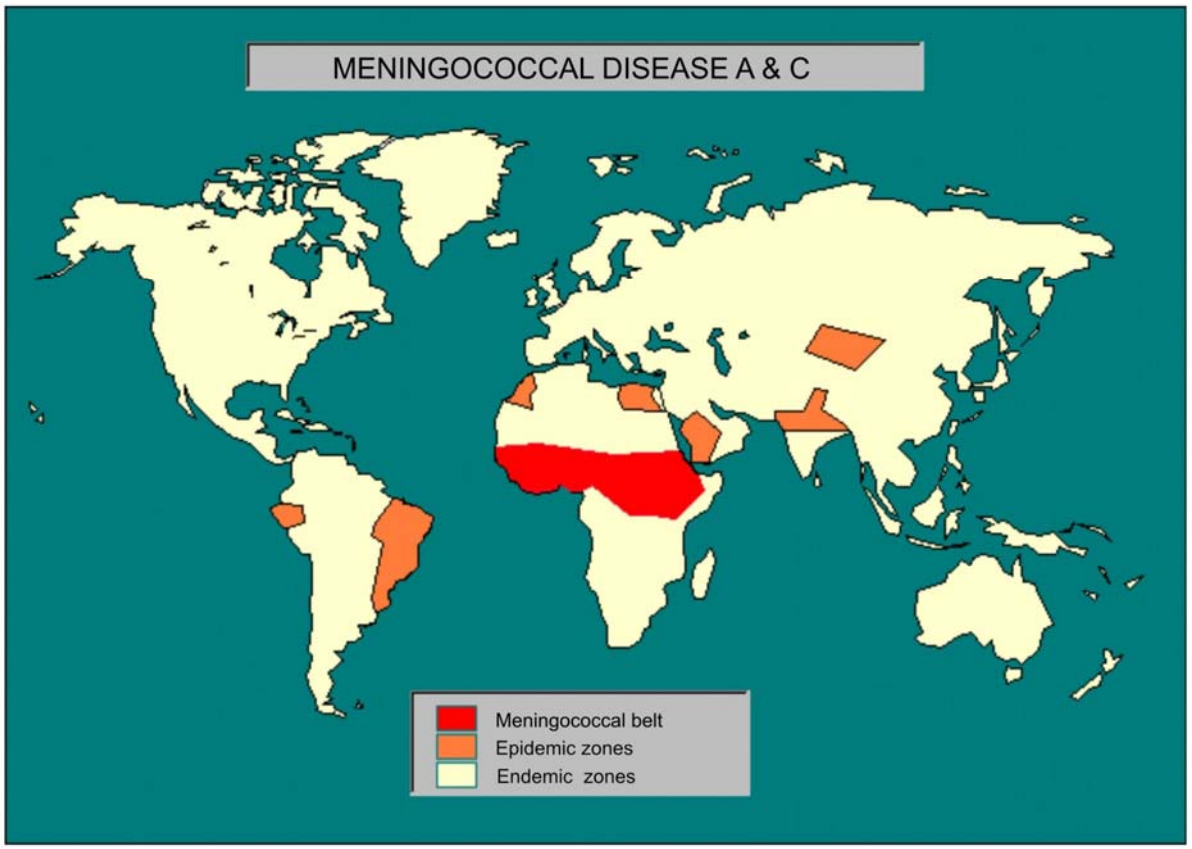

Figure 2. Meningococcal disease in the world

The area with frequent epidemics of meningococcal meningitis (sub-Saharan zone) is evidenced in red.

MENINGOCOCCAL DISEASE: PHYSIOLOGY AND PATHOGENESIS

Protective antibodies may be acquired either passively by transplacental passage of immunoglobulins or actively as the result of oropharyngeal infection with both capsulated and noncapsulated meningococci (Goldschneider, et al., 1969; Reller et al., 1973). Moreover, one cannot rule out subclinical systemic infections as a mechanism for active immunization. The effect of passively acquired immunity is relatively short lived but is effective in protecting infants against meningococcal disease, as evidenced by the low incidence of cases in the first 6 months of life (Smith, 1954). The susceptibility to disease increases progressively during the first 6 months of age (Goldschneider, et al., 1969), which correlates with the progressive decrease in bactericidal activity of sera. The lowest levels of serum bactericidal activity are found between 6 months and 2 years of age, which accounts for the high incidence of meningococcal disease within this age group. Moreover, the progressive reduction in the incidence of disease after 24 months of age correlates with the rise in serum bactericidal activity in children, with adult levels of antibodies developing by the age of 10 years (Frasch, 1977).

Protection against meningococcal disease may also result from exposure to cross-reactive antigens from bacteria which bear little or no taxonomic relationship to meningococci. For example, the capsular polysaccharide from *Escherichia coli* strain O7:K1(L):NM is immunologically and chemically indistinguishable from that of the group B meningococcus (Kasper et al., 1973). Furthermore, *Bacillus pumilis* antigens cross-react with those of group A meningococci (Robbins et al., 1974).

The characteristics of the strains of *Neisseria* carried by adult and children differ markedly. The reasons for this difference are not readily apparent but may stem from the variation between adults and children in the oropharyngeal surface environments, i.e., availability of microbial nutrients, nature of microbial attachment sites on hot cell surface, etc.

According to all data currently available the meningococcus can establish a systemic infection only in human hosts that lack serum bactericidal antibodies directed against capsular or noncapsular antigens of the invading strain, or in patients deficient in certain complement components (Frasch, 1979; Goldschneider, et al., 1969). That the bactericidal activity in serum of immune hosts is complement mediated is evidenced by the repeated episodes of meningococcal infections in patients lacking the higher complement components C5, C6, C7, and C8, which are required for immune bacteriolysis (Lee et al., 1978; Lee et al., 1979; Lim et al., 1976; Nicholson & Lepow, 1979; Petersen et al., 1979). Moreover, those deficient in IgM appear to be at greater risk from meningococcal infections than normal hosts (Hobbs et al., 1967; Jones et al., 1973). The IgM antibody has been identified as the primary one involved in immune bacteriolysis of gram-negative bacteria (Evans et al., 1966; Michael et al., 1962; Michael et al., 1963).

The meningococcus is thought to enter the systemic circulation directly from the oropharynx. The establishment of growth in the deeper tissues or systemic circulation of the susceptible host follows the onset of carrier acquisition by only a few days and only rarely do susceptible patients have a prolonged oropharyngeal carriage before the onset of overt signs of disease (Artenstein, 1967; Murray, 1929). The invasion of the blood stream is preceded by an overt oropharyngitis in about one-third of the cases, but the role of the meningococcus as a causative agent in pharyngitis has not been determined (Bauks, 1948).

Once the meningococcus has entered the systemic circulation of the susceptible host and has established a population, one of the several faces of meningococcal disease will make itself manifest. Upon the primary colonization of the oro- or nasopharynx (carrier state) the bacterium enters the blood stream and gives rise to meningococcemia. Concomitantly with the bacteremia, or at various times thereafter, the meningococci metastasize to the skin, eyes, joints, heart, adrenals, or meninges and produce various clinical symptoms (Fig. 3). In 90% of

cases, meningococcal disease will progress to an inflammation of the leptomeninges (pia and arachnoid) with associated dermal lesions (petechiae or the larger purpura) in approximately half the cases (Swartz & Dodge, 1965).

The neisserial infection is a multifactorial process, and involves a series of receptor-mediated interactions between the bacteria and the primary target cells. The formation of pili is a prerequisite for attachment to the epithelial cell surface. The surface-attached bacteria often penetrate the epithelial cells and, whilst contained in a vacuole, may transcytose towards subepithelial tissues. The pathogenic *Neisseriae* strongly interact with phagocytic cells, such as neutrophils and macrophages, which seem to provide them with an intracellular habitat (Meyer et al., 1994).

The organism is capable of adapting to growth in a variety of host environments. Moreover, the clinical response of the host to meningococcal invasion can vary from a relatively benign picture to the extreme of fulminating meningococcemia with death in hours. The microbial mechanisms in the pathogenicity of the various forms of meningococcal disease are complex and well adapted to evading the host responses to the invading microbe.

In their simplest form the requirements for microbial survival upon entry into body fluids or tissues do not differ greatly from those any microbe faces in a new environment except that the animal host has a preprogrammed response against invading organisms. Successful pathogens, such as the meningococcus, must have mechanisms to overcome such responses of the host. The fatality rate in meningococcal disease before the age of antibiotic therapy was sufficiently high (75 to 90%) for one to conclude that the meningococcus does posses the required mechanisms to overcome or evade all host defences (DeVoe, 1982). Most literature treats virulence of the meningococcus as fixed property in given strains. Virulent strains are considered to be those which in low numbers cause disease in some animal model, whereas avirulent strains are those that are either incapable of producing disease in an animal model or

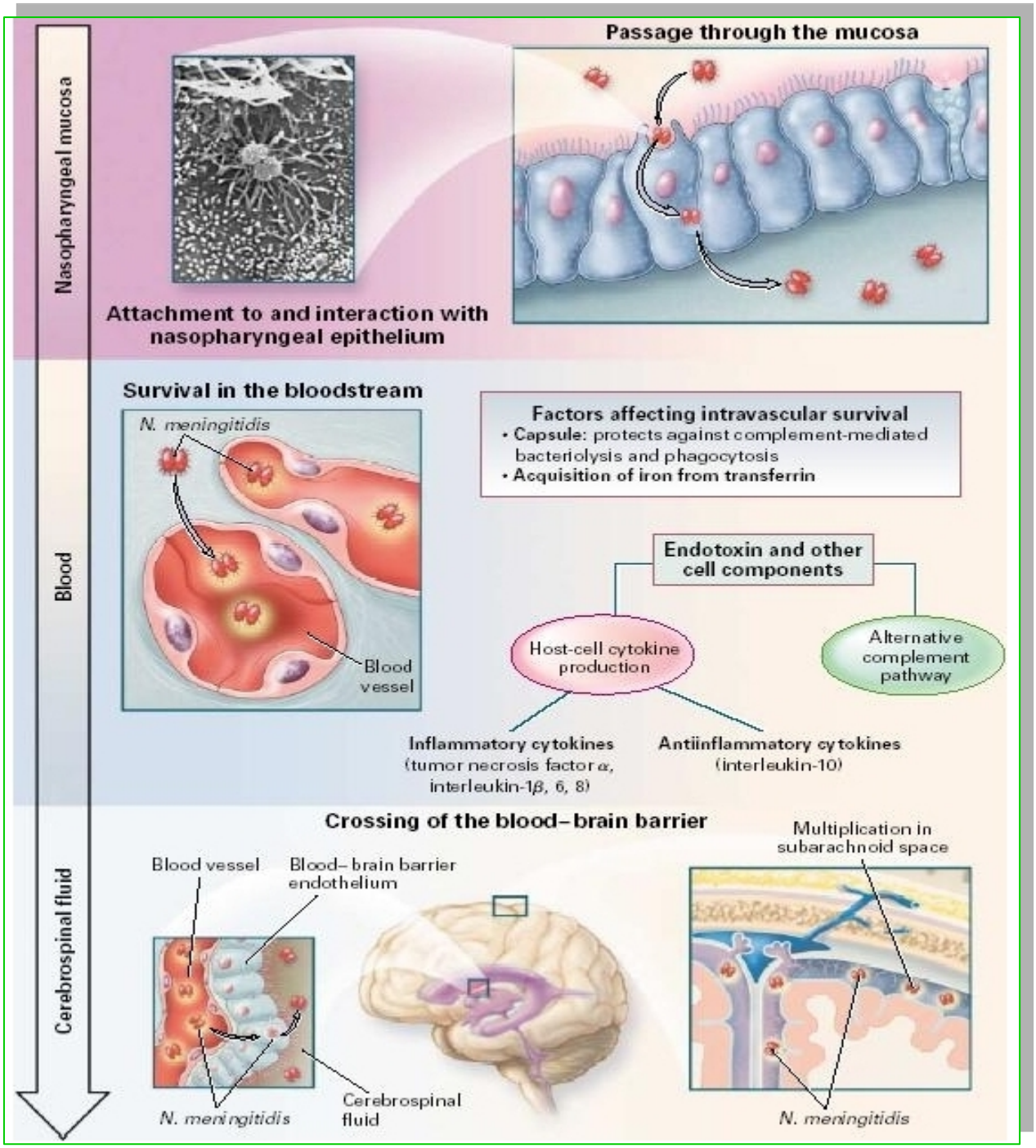

Figure 3. Infection cycle of *Neisseria meningitidis*

After colonizing the nasopharynx, the bacteria adhere to pili and cross the nasopharyngeal epithelium by a process of endocytosis to invade the circulatory system. In the bloodstream, the bacteria proliferate and adapt to the host environment. Clinical responses to infection are varied from a benign form to extreme, fatal forms (meningococcaemia). In its most severe form, heightened levels of bacteria in the blood make their way to the meninges in the brain and at such high levels of cytotoxicity, induce inflammation of the meninges and progression of the disease.

require relatively large numbers of organisms to do so. The observation that most hosts can harbour "virulent" serotypes of meningococci in the oropharynx but show no overt signs of disease is interpreted in the framework of a strong host defence (bactericidal antibody) specifically directed at, or cross-reacting with, the meningococcus. On the other hand, during epidemics only certain serotypes are routinely isolated from diseased patients, suggesting that such microbes possess certain fixed virulence properties. The evidence in support of both serum bactericidal host resistance and the virulence associated with specific serotypes is convincing and has gained general acceptance. In contrast, the spectrum of manifestations of meningococcal disease during epidemics has not been adequately explained. It is possible that certain patients have little or no defence whatever against the invading meningococci and, therefore, fall victim to fulminating meningococcemia, whereas others with stronger defences have less severe forms of the disease. The past histories of those with fulminating disease do not support the conclusion, however, that they were unable to defend against bacterial infections in general. Therefore, one must consider either a transitory anergy, such as that brought on in some patients by surgery or traumatic injury (McLoughlin et al., 1979), or a more virulent organism that can evade host defences, obtain required nutrients from the host more efficiently, or both. There is no evidence to support a transient state of anergy associated with the onset of meningococcemia. However, there is evidence that environmental changes around the meningococcus can greatly influence the metabolism and phenotypic expression of the organism. Brener et al. grew a strain of meningococcus at its lowest tolerable pH (6.6) and under conditions of iron starvation. The phenotype of the meningococci grown at low pH and limited for iron in Mueller-Hinton broth changed dramatically as compared to those grown under routine iron-sufficient conditions at neutral pH. The relative virulence index for the mouse increased 1,200-fold from a 50% lethal dose of 3,600 to one of 4 organisms. In vitro experiments showed the bacteria to be resistant to phagocytosis by both mouse and rabbit

PMNs, whereas the same strain grown conventionally was readily phagocytosed (Brener et al., 1981).

There is also some indirect evidence for meningococcal phenotypic shifts in vivo. Griffiss et al. reported a recurrent infection in a child who developed bactericidal antibody against the invading strain. In the recurrence the serotype and serogroup were identical, leading the authors to suggest that in vivo alterations in the meningococcus were the result of environmental changes in the host (Griffiss et al., 1974).

There are, no doubt, other phenotypic changes that occur as a result of iron starvation or low pH or both. Cells grown at low pH may be more stable and less susceptible to autolysis because the peptidoglycan in less prone to hydrolysis at low pH (Wegener et al., 1977). However, the most dramatic phenotypic change expressed after growth of meningococci in iron-limited media was the expression of a highly efficient mechanism to remove iron from human transferrin, the only ready source of iron in the blood (Simonson et al., 1982). The metabolic responses of pathogenic *Neisseriae* to the low pH and low iron are substantial. These changes would contribute to the survival potential in the host. It would appear that the organisms respond metabolically to the inflammatory reaction in the host by becoming stronger and more virulent.

MENINGOCOCCAL DISEASE: PREVENTION AND PROPHYLAXIS

A polysaccharide vaccine against disease by *N. meningitidis* serogroups A, C, Y, and W-135 is currently licensed. The vaccine consists of 50 μg of the respective purified bacterial capsular polysaccharides (Meningococcal vaccine. MMWR, 1985). The serogroup A polysaccharide induces antibody in some children as young as 3 months, although a response comparable to that seen in adults is not achieved until 4 or 5 years of age; the serogroup C component does not induce a reliable antibody response before 2 years of age. A booster response after a second dose occurs with group A vaccine but not with group C vaccine (Lepow et al., 1986). Serogroup A vaccine has been shown to have a protective efficacy of 85% to 95% and to be useful in controlling epidemics. A similar level of clinical efficacy has been shown for the serogroup C vaccine. The groups Y and W-135 polysaccharides have been shown to be safe and immunogenic in adults and in children older than 2 years. Antibodies, against the group A and C polysaccharides, decline over the first three years following a single dose of vaccine. This antibody decline is more rapid in infants and young children than in adults.

Except for the military population, routine vaccination with a meningococcal polysaccharide vaccine is not recommended because the risk of infection is substantially low; a vaccine against serogroup B, the major cause of meningococcal disease is not yet available; and much of the meningococcal disease occurs among children too young to respond to the current vaccines. In an outbreak of meningococcal disease, however, the serogroup should be identified and the population at risk determined. Older children, adolescents, and young adults at high risk may warrant vaccination if the outbreak is caused by one of the serogroups included in the vaccine (Meningococcal vaccine. MMWR, 1985).

Routine immunization with the quadrivalent vaccine (groups A, C, Y, and W-135) may be indicated for certain immunodeficient patients at high risk for meningococcal disease, such as

patients with specific complement deficiencies and anatomic or functional asplenia, although clinical efficacy has not been documented. The use of the vaccine should be considered to control outbreaks of disease caused by serogroups represented in the vaccine, and its use may be of benefit to travellers to countries recognized as having hyperendemic or epidemic disease as well as to persons living in epidemic areas who will have prolonged contact with the local population. The vaccine is currently given to all recruits. For both adults and children, vaccine is administered subcutaneously as a single 0.5 ml dose. Good antibody levels are achieved within 10 to 14 days. Specific immunity occurs from a single dose in 89% to 100% of vaccinations (Fulginiti, 1985). The duration of immunity is not known, and the need for booster doses remains to be determined, but revaccination after two or three years may be indicated for persons at high risk of infection, particularly children who were first immunized before 4 years of age. The meningococcal vaccines appear to be safe; localized erythema may occur and last one to two days, but no serious adverse effects are known.

Antimicrobial chemoprophylaxis of intimate contacts remains the primary preventive measure in sporadic cases of *N. meningitidis* disease because as many as half of the secondary cases of meningococcal infection occur after five days. Chemoprophylaxis using either rifampin or minocycline hydrochloride has been recommended; sulfonamides may be used if the infecting strain is known to be susceptible. Household, day-care centre and nursery school contacts should receive antibiotic prophylaxis as soon as possible, preferably within 24 hours of the diagnosis of the primary case. Chemoprophylaxis is not routinely recommended for medical personnel except for those with intimate exposure. Systemic antimicrobial therapy for meningococcal disease does not reliably eradicate nasopharyngeal carriage of *N. meningitidis* (Fulginiti, 1985).

INFECTION FROM A CELL BIOLOGY POINT OF VIEW: PATHOGENIC *NEISSERIAE* AND DETERMINANTS OF VIRULENCE

The pathogenic *Neisseriae* produce diverse molecules (determinants of virulence) that can interact with host cells. However, the expression of these determinants varies widely among the members of an infecting population, even if the population begins as a clonal isolate. Two general types of variation occur. In phase variation, the control of expression is binary: on or off. In antigenic variation, the variant protein changes in primary sequence. Both types of variation occur through hereditable genetic mechanisms, which have been studied intensively and are reviewed subsequently.

The *Neisseriae* initiate infection by attaching to host cells via surface-associated filaments called type IV pili. The pili, fine, hair-like organelles protruding from the bacterial cell surface, probably represent the most variable structures produced by the pathogenic *Neisseriae*. In the bacterial genome multiple gene copies exist most of which are unexpressed, incomplete (silent/cryptic) gene copies (*pilS*), while only one or two of these represent the expressed gene copies (*pilE*). The *pilS* copies constitute the variant sequence repertoire which is used for recombination with *pilE* to generate variant pilin molecules. Owing to this remarkable variability, the bacterial pili can effectively escape the human immune response, since the variability of antibodies and T cell receptors of the immune system, which uses a similar variation mechanism, is within the same order of magnitude. Recent studies have provided evidence that recombination of *pil* genes can occur by at least four pathways, i.e., reciprocal, nonreciprocal (gene conversion-like), transformation-mediated recombination and deletion of duplicated gene copies (Haas and Meyer, 1986; Manning et al., 1991; Swanson et al., 1986; Seifert et al., 1988; Gibbs et al., 1989; Hill et al., 1990; Zhang et al., 1992; Facius & Meyer, 1993). The pili are an absolute requirement for the initiation of an infection (Kellogg et al., 1968) in that they confer the attachment of the bacteria to epithelial cells (McGee et al., 1981;

Stephens & McGee, 1981). The binding of pili appears to be specific for human cells and the pili therefore represent a major determinant of the neisserial species tropism. Owing to their exposed location they are also strong targets for an antibody response that could interfere with receptor recognition.

Early studies suggested a direct role for pilin (PilE), the major subunit, in adherence to various cellular substrates (Schoolnik et al., 1984; Virji & Heckels, 1984; Rothbard et al., 1985). While this possibility still exists, recent studies provide evidence for different adherence properties of the pili. At least three distinct adherence specificities, one for epithelial cells, one for erythrocytes and one allowing intergonococcal adherence, can be distinguished, either genetically or otherwise (Rudel et al., 1992). This does not exclude the association of pili with yet other adherence properties, such as for endothelial, phagocytic and sperm cells. Furthermore, *N. meningitidis* can produce two different classes of major subunit, class I and class II pilins, which may affect the adherence properties (Virji et al., 1989; 1992a). Pilin can be modified by phosphorylation and /or glycosylation (Robertson et al., 1977; Schoolnik et al., 1984; Virji et al., 1993b), and it has recently been shown that the variant-dependent glycosylation of gonococcal pilin can influence receptor recognition (Virji et al., 1993b). However, the observed effect of pilin variation on adherence may be indirect and does not preclude a role for potential minor subunits in adherence.

Indeed, recent studies suggest an association of minor protein components with the gonococcal pilus (Muir et al., 1988; Parge et al., 1990). One of these components, the PilC protein, has been characterized genetically and implicated in the biogenesis of pili (Jonsson et al., 1991), in epithelial cell adherence (Rudel et al., 1992) and transformation competence (Rudel et al., 1995). Type IV pili also, mediate neisserial motility: twitching motility and social gliding motility, modes of migration along liquid-solid or liquid-air interfaces, implicated in a wide array of biological processes (Wall & Kaiser, 1999; Pujol et al., 1999; Merz & So, 2000).

Nonetheless, pili can be assembled in the absence of either of the two known PilC1 and PilC2 proteins, probably by utilizing an alternative PilC-like assembly factor (Rudel et al., 1992). Moreover, the pili of pathogenic *Neisseria* species, irrespective of their structural variability, recognize the same, or a closely related, group of receptors. The first receptor for neisserial pili, CD46, was identified only recently (Källström et al., 1997). CD46, or membrane cofactor protein (MCP), is a member of the superfamily of complement resistance proteins. At least six CD46 splice variants have been identified, and CD46 variants are expressed on nearly all human cells except erythrocytes.

The second major class of neisserial adhesins, the Opaque (Opa) proteins (previously referred to as class V proteins of the meningococcus and P.II proteins of the gonococcus), is encoded by the multigene *opa* family. *N. gonorrhoeae* strains typically harbour 11 *opa* loci, whereas *N. menigitidis* strains have 4-5 *opa* loci (Dehio et al., 1998). Specific *opa* repertoires vary from strain to strain. In contrast to the *pilE* genes, the genes encoding Opa proteins represent complete, rather than incomplete, variant genes (Stern et al., 1986; Jonsson et al., 1991) which undergo frequent phase transitions (on/off switches) but rarely recombine with each other (Connell et al., 1988; Bhat et al., 1991). These switches occur via a DNA slippage mechanism involving homo- or heteropolymeric repeated nucleotide sequences within the coding sequences of the genes (Connell et al., 1988; Robertson & Meyer, 1992); variation of the number of the repeating units alters the reading frame and consequently affects the translation into functional gene products. In case of the *opa* genes, the repeating unit is a pentameric (CTCTT) sequence.

The role of Opa proteins in various adherence functions, such as interbacterial adhesion and interaction with human epithelial and phagocytic cells, has long been recognized (King & Swanson, 1978; Lambden et al., 1979; Virji & Heckels, 1986; Fisher & Rest, 1988; Makino et al., 1991; Belland et al., 1992; Virji et al., 1993a). Recent works suggest that Opa proteins not

only cause bacterial adherence but also trigger important cellular functions: the epithelial cell invasion (Virji et al., 1993a) and polymorphonuclear cells (PMNs) stimulation (Kirchner et al., 2005).

The Opa protein-host cell interaction seem to be a two step event, a weak and reversible attachment conferred by most of the Opa proteins and a tight probably receptor-specific interaction, this latter form appears to be a prerequisite for entry into host cells (Bessen & Gotschlich, 1986; Makino et al., 1991). A number of signalling molecules serve as receptors for various Opa proteins. The binding of Opa variants to their cognate receptors initiates different signalling cascades, leading to entry of bacteria vie distinct pathways, possibly also to different intracellular fates and/or infection outcomes. The first class of Opa receptors is heparan sulphate proteoglycans (HSPGs), which bind a subset of Opa variants that have surface-exposed loops rich in positively charged amino acyl residues (Chen & Gotschlich, 1996; van Putten & Paul, 1995; Virji et al., 1999). Opa-HSPG interactions are not sufficient to promote internalization in epithelial cells; thus the possibility remains that Opa-HSPG interactions mediate only non-specific adhesion and that porins or other molecules then trigger lipid hydrolysis and bacterial uptake (van Putten et al., 1998a). Recent work from several groups has shown that many different Opas bind to members of the large CD66-related family of proteins. Different CD66 family members are variably expressed on different cell types. Depending on the particular CD66 variant and Opa protein expressed, different host cell responses occur, including binding, uptake, and the activation of different signal transduction pathways (Bos et al., 1997; Chen & Gotschlich, 1996; Chen et al., 1997; Gray-Owen et al., 1997a, b; Hauck et al., 1998; Virji et al., 1996 a, b, 1999; Wang et al., 1998).

The pathogenic *Neisseriae* often carries a copy of the phase variable *opc* gene whose product has a function similar to the epithelial cell-specific Opa proteins (Virji et al., 1992b). It is thus

evident that the variable Opa and Opc adhesions represent important cell tropism determinants of *Neisseria* spp and that the variability of these proteins allows multiple cellular interactions.

The *Neisseria* species produce a short type of lipopolysaccharide (LPS) which lacks any repetitive O-side chains. Nonetheless LPS preparations from in vitro cultured bacteria reveal multiple size classes indicating a structural heterogeneity of the neisserial LPS (Schneider et al., 1988). Several lines of evidence suggest that LPS variation also occurs in vivo. In meningococcal carriers, the majority (70%) of bacteria isolated from the nasopharynx are unencapsulated and preferentially express a short LPS species (Broome, 1986), whereas in the diseased state, 97% of the blood and CSF isolates are encapsulated and of the long LPS species (Jones et al., 1992). A major difference among the variant LPS molecules is the presence of additional carbohydrate residues in the longer LPS forms that can be externally modified by a membrane-associated bacterial sialyltransferase using host-derived or endogenous cytidinemonophosphate-N-acetylneuraminic acid (CMP-NANA) as sialyl donor (Smith,1991; Mandrell & Apicella, 1993; van Putten, 1993). The functional relevance of the LPS phase transition has recently been elucidated and appears to lie in the expression of variable amounts of sialic acid incorporated in the different forms of LPS. A low sialylation phenotype, as found early in the infection, enables entry of the bacteria into mucosal cells, but makes them susceptible to bactericidal activity. In contrast, highly sialylated bacteria are incapable of entering epithelial cells but are resistant to phagocytosis and killing by antibodies and complement, allowing persistence of infection. Thus depending on the degree of sialylation the bacteria are either adapted to the extracellular environment and are capable of resisting humoral immune mechanisms, or they become sensitive to bactericidal activities but then readily enter the intracellular milieu via Opa-mediated cellular interaction (van Putten, 1993; Schneider et al., 1991).

The function of highly sialylated LPS has many similarities with that of polysialic capsules produced by *N. meningitidis* and many commensal *Neisseria* species, in that they protect extracellular bacteria against both specific and non-specific host responses (Frosch et al., 1989).

The polysaccharide capsule is the major pathogenicity factor of meningococci. Capsule expression is dependent on the possession and expression of the genes for capsule biosynthesis, modification, and transport, which are located in the regions A, B, and C, respectively of the *cps* locus of the meningococcal genome (Frosch et al., 1989) (Fig.4). The genes in region A determine the biochemical composition of the capsule and hence the serogroup (Edwards et al., 1994; Claus et al., 1997; Swartley et al., 1997). In meningococci expressing capsules containing sialic acid (those corresponding to serogroups B, C, W-135, and Y), the region A comprises the genes *siaA, siaB,* and *siaC,* which are necessary for the synthesis of activated sialic acid (CMP-Neu5Ac), and the serogroup-specific gene *siaD* encoding the polysialyltransferase. In vitro studies have shown that slipped strand mispairing, as the reversible +1/-1 frameshift mutation within a poly (dC) repeat (Lavitola et al., 1999), and insertions into this region of the mobile element IS1301 account for reversible inactivation of the serogroup B capsule expression (Hammerschmidt et al., 1996a,b) which may favour colonization of the nasopharynx. Although phase variation of capsule expression has been observed, this structure appears to be primarily regulated by environmental factors (Brener et al., 1981). Loss of capsule expression favours an Opa and Opc-mediated interaction of *N. meningitidis* with target cells and thus seems to be a prerequisite for the cellular invasion (Virji et al., 1993a). The importance of the role of capsule in pathogenesis and as a vaccine component has stimulated interest in understanding the genetic mechanisms for regulation of its expression in the human host. Therefore, recent advances made in the analysis of capsular

(*cps*) gene organization and functions are important for understanding of the conditions favouring invasive meningococcal infection.

The porins, abundant proteins of the outer membrane, form anion-selective ions channels that are essential for neisserial viability (Barlow et al., 1989; Gotschlich et al., 1987; Murakami et al., 1989; Suker et al., 1994). The functions of porins in colonization remain unclear but over the last 20 years several findings have suggested that these proteins exert a pivotal influence on the outcome of host-bacterial interactions. Some evidences indicates that porins (PorA and PorB) are capable of translocating into host cell membranes as active, gated ion channels, favouring the host cell invasion (Lynch et al., 1984; Mauro et al., 1988, Mietzner et al., 1987; Rudel et al., 1996). It interferes with cell signalling, inducing a transient membrane hyperpolarization, inhibition of phosphatidylcholine-phospholipase C activity and failure of the NADPH oxidase function (Haines et al., 1988; Haines et al., 1991; Lorenzen et al., 2000).

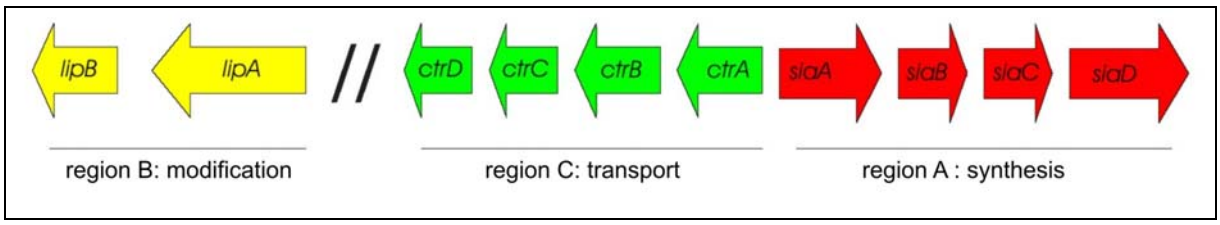

Figure 4. Schematic depiction of the capsule locus *cps* of *N. meningitidis*

The genes for capsule biosynthesis, modification, and transport are located in the regions A, B, and C, respectively of the *cps* locus of the meningococcal genome. The genes in region A determine the biochemical composition of the capsule. In meningococci expressing capsules containing sialic acid, the region A comprises the genes *siaA, siaB,* and *siaC,* which are necessary for the synthesis of activated sialic acid, and the serogroup-specific gene *siaD* encoding the polysialyltransferase.

A common feature of pathogenic *Neisseriae* is the production and secretion of an antigenically diverse family of endoproteinases specifically affecting immunoglobulin A1 (IgA1) of their human hosts (Plaut et al., 1975). Like the closely related enzymes produced by *Haemophilus influenzae* these IgA proteases belong to the class of serine proteases (Bachovchin et al., 1990). Unlike the pilus and Opa proteins, IgA proteases are encoded by single copies of *iga* genes. Horizontal exchange of *iga* sequences resulted in numerous serologically distinct IgA proteases in *N. gonorrhoeae* and *N. meningitidis* (Halter et al., 1989; Lomholt et al., 1992; Morelli et al., 1994). Despite this sequence variability the key features of the *iga* genes and gene products are conserved (Halter et al., 1989). Recent works have shown that the IgA proteases cleave Lamp1 (*lysosome-associated membrane protein*), abundant transmembrane glycoprotein of mammalian lysosomes, favouring the survival of bacteria in the epithelial cells (Lin et al., 1997; Ayala et al., 1998).

As for most other pathogens, the molecular basis of infectious process events is a current topic in *Neisseria* research. Several evidences indicate that the initial attachment of *Neisseria* to mucosal cells may be followed by ingestion of attached organisms when the appropriate phenotype is present. Morphologically, this process involves an elongation of the microvilli and, in order to encompass the bacteria, the formation of zones of intimate contact between the bacterial and host cell membranes, along with engulfment of the bacteria via a zipper-like mechanism (Watt & Ward, 1980; Tjia et al., 1988; Well & van Putten, 1988; Well et al., 1991a, b; Stephens, 1989). It has been speculated that the insertion of the major neisserial ion channel protein I (porins) into host cells is a critical determinant for entry (Blake, 1985; Well & van Putten, 1991; Well et al., 1991b). Immunomorphological evidence supports binding to, or insertion, the proteins into the host cell membrane at the sites of bacterial entry during infection (Well & van Putten, 1991; Well et al., 1991b). Complete internalization of the bacteria requires phosphorilation of host proteins at tyrosine residues, but whether Opa protein,

porins, and other bacterial factors trigger this event is unclear (Grassmé et al., 1996). In all cases, it should be emphasized that these observations may be unique to the infection system and bacterial strain used, and may not represent uniform mechanisms or reflect the in vivo situation.

Neisserial colonization of the human mucosa may give rise to an inflammatory response with recruitment and activation of professional phagocytes and activation of the host immune defence. Morphological data suggest that, in natural infection, the pathogenic *Neisseria* may reside inside phagocytes (Ward et al., 1972; DeVoe et al., 1973; Swanson & Zeligs, 1974). Dissection of the immunoresistant phenotype indicates that the bacteria possess a variety of antiphagocytic properties as well as a large repertoire of mechanisms that potentially allows them to be facultative intracellular microorganisms. An important step in *Neisseria* phagocyte interaction appears to be the distinct Opa protein-associated contact with the cells which results in induction of a respiratory burst and which may be followed by ingestion of the bacteria (King & Swanson, 1978; Rest et al., 1982; Virji & Heckels, 1986; Fisher & Rest, 1988; Belland et al., 1992).

Once ingested, the bacteria probably reside in phagosomes and are exposed to the extremely hostile environment of oxidative and nonoxidative killing mechanisms, unless phagolysosomal fusion is inhibited, as may occur by the insertion of the bacterial ion channel in the vesicle membrane (Well et al., 1991b; Well & van Putten, 1991). The bacteria may counter the oxidative killing by increased oxygen consumption (Krieger et al., 1980; Cohen & Cooney, 1984; Britigan et al., 1988), the production of high levels of catalase (Archibald & Duong, 1986; Johnson et al., 1993) and by switching to anaerobic growth using nitrite as a source of energy (Hassett & Cohen, 1989). In addition, expression of a long LPS type and intracellular bacteriostasis may promote resistance to nonoxidative antimicrobial agents either by preventing the binding of cathepsin or antimicrobial cationic proteins to the bacterial penicillin

binding protein or inhibiting their detrimental effect, which requires active cell wall synthesis (Daly et al., 1982; Casey et al., 1985; Rock & Rest, 1988; Shafer; 1988; Shafer et al., 1990). In this way, the nongrowing bacteria may survive for prolonged periods awaiting cytotoxic lysis or exocytotic release from the cells perhaps as clumps of multiplying bacteria enclosed in host cell remnants. These so-called infectious units have been proposed to play an important role in the persistence and transfer of neisserial infection (Novotny et al., 1977).

EVOLUTIONARY CONTEXT AND HORIZONTAL EXCHANGE BETWEEN *NEISSERIA* SPP

A series of recent studies emphasized the significance of horizontal genetic exchange in the evolution and epidemiology of *Neisseria* species. It appears that a continuous horizontal flow of genetic material affects the chromosomal composition not only of the pathogenic *Neisseria* species, but also of many commensal species (Halter et al., 1989; Manning et al., 1991; Feavers et al., 1992; Spratt et al., 1992; Zhou & Spratt, 1992). It is difficult to give precise estimates of the frequencies with which such horizontal exchanges occur in nature, however, in a few cases it is plausible that such events may have happened in recent years (Achtman, 1994). Conceivably, horizontal genetic exchange can be envisioned as a long-term adaptive mechanism suitable for responding to gross environmental changes and for securing the genetic flexibility of *Neisseria* species.

Smith and colleagues (Smith et al., 1991) proposed, based on gene linkage disequilibrium measurements, a significant difference in the clonal structures of *N. meningitidis* vs. other clonal species, such as *Salmonella* and *Escherichia coli*. The authors define several types of population structures, an entirely clonal structure, the epidemic structure and, at the other extreme, the panmictic structure with no evidence of linkage disequilibrium. These population structures are the result of two competing genetic processes, the genetic drift (mutagenesis) occurring within the scope of a species, and horizontal exchange (recombination) affecting this species extrinsically. *Salmonella* is little influenced by horizontal exchange and thus appears as a clonal population. Contrastingly *N. gonorrhoeae* constitutes a panmictic population in which horizontal exchange is overwhelming (O'Rourke & Stevens, 1994). *N. meningitidis* occupies an intermediate position in which occasional clonal outgrowths occurs in an otherwise

panmictic context. The latter population structure could be termed a "clonal network" comprising both sexual and clonal elements.

The striking contrast between the strict clonality of *Salmonella* and *E. coli* and the clonal network structure of *Neisseria* appears to be reflected by distinct differences in the chromosomal organization of the genes of these species. The *E. coli* chromosome exhibits a considerable degree of organization concerning, for example, the linkage of related genes, the presence of operons and the transcriptional orientation of genes. Such a high degree of genomic organization is not seen in *Neisseria*, in which many functionally related genes (i.e., *pil* and *opa* genes) are distributed over the genome. Also, transcriptional coupling of genes (i.e., operons) is rarely found in *Neisseria*, although a few exceptions exist, including the tightly organized *cps* capsular gene cluster of the meningococcus (Frosch et al., 1989). Another remarkable phenomenon of *Neisseria* is the occurrence of multiple gene copies (gene families) which usually contain significantly diverse sequences. Whether the distinct chromosomal organization of *Neisseria* species is causally related to other unique features of these microorganisms, including the natural transformation competence, intra-strain variability and the population structures, is not well understood. Above all, it is unknown whether the genetic plasticity found in *Neisseria* species is related to the life style of these organisms, characterized by an extremely narrow host range and the ability to cause persistent infections, or whether it simply reflects the fact that these bacteria are young in evolutionary terms and still at the beginning of a long-term adaptative process with regard to their host.

The ability of *Neisseria* spp to undergo DNA transformation under natural conditions was recognized some 30 years ago (Catlin & Cunningham, 1961; Sparling, 1966). Natural transformation competence differs markedly from the artificial transformation used for the cloning of genes, for example, in *E. coli*. In *Neisseria* the transforming DNA is taken up as a linear molecule and requires the RecA function and homologous sequences in the resident

DNA of the recipient cell in order to allow recircularization of a plasmid or incorporation of the DNA into the chromosome (Biswas et al., 1986; Koomey et al., 1987). The DNA homology requirement is probably the most effective mechanism of protection against bacterial transformation with unrelated DNA. Whether there is any role for the abundant DNA restriction and methylation systems of *Neisseria* species (Sullivan & Saunders, 1989; Gunn et al., 1992; Gunn & Stein, 1993) on transformation is currently not well understood. In addition to these restrictions, the *Neisseria* species recognize a specific DNA sequence, 8 bp in length that serves as a signal for the efficient uptake of DNA (Goodman & Scocca, 1988).

HYPERMUTATION IN PATHOGENIC BACTERIA: STRATEGIES FOR RAPID MICROENVIRONMENTAL ADAPTATION

Microbial populations not only need to adapt to the gross long-term environmental changes encountered during co-evolution with the host but, in addition, encounter frequent usually recurrent microenvironmental changes, for example, during the course of an infection. In order to respond to such recurrent changes, microorganisms maintain retrievable genetic programs. There are two principle types of adaptive programs used by microorganisms: (1) Genetic variation concerns spontaneous changes in the DNA which are inherited to the progeny and are often reversible. These changes, although temporally random, take place at spatially distinct loci and ultimately lead to the synthesis of altered gene products. As a consequence, genetic variation generates heterogeneous population of a distinct microbial strain, such that a fraction of this population is likely to show an improved microenvironmental adaptation. (2) By contrast, the second microbial adaptation strategy, gene regulation, influences the bacterial population as a whole. In response to a certain environmental stimulus, such as temperature, osmolarity, or specific substances, the bacteria alter the expression of responsive genes in a coordinated fashion. Stress responsive systems sensitive to heat shock and/or other stress conditions, such as nutrient starvation and iron limitation, have been identified. The genetics of some of these systems is currently being studied (Taha et al., 1988; Fyfe et al., 1993).

Obviously both strategies have specific advantages for microorganisms: while genetic variation better protects minor parts of the population against a large variety of unpredictable changes, gene regulation affects a well-determined adaptive process for the benefit of the whole population. However, genetic variation and gene regulation are not exclusive and are often interconnected. Likewise, the frequency and the direction of a switch may be influenced by

environmental effectors, and conversely, a phase-variable regulator protein may control the expression of genes (Robertson & Meyer, 1992).

Several works indicate that the genes coding for virulence determinants, in *N. meningitidis* and many so-called pathogenic bacteria such as *Haemophilus influenzae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, must evolve in response to the demands (selection pressures). *N. meningitidis*, like other commensal bacteria, must surmount a variety of environmental hurdles to successfully colonize a genetically and immunologically diverse host population, evade the constitutive and inducible defences of these hosts and be transmitted to new hosts. To meet these challenges, these bacteria have highly mutable "contingency" genes (Moxon et al., 1994; Bayliss & Moxon 2002), which code over 100 surface-associated components, including capsule, type IV pili, and surface sugar biosyntesis proteins involved in lipooligosaccharide metabolism and outer membrane proteins acting as porins, adhesins, or invasins or involved in iron uptake (Moxon et al., 1994; Snyder et al., 2001). Mutations in these genes, coding for molecules needed for colonization and persistence in human hosts and transmission to new hosts, generate variation and ensure that at least a fraction of a bacterial population expresses a phenotype that is compatible with a productive infection cycle (Hammerschmidt et al., 1996; de Vries et al., 1996).

In the case of *N. meningitidis,* contingency genes are turned on and off by a mutational mechanism called phase shifting (or phase variation) that increases the rate at which these genes mutate to different states, including states that may be detrimental to the bacterium. Depending on the strain, the *N. meningitidis* contains a different number of these contingency genes, each of which contains microsatellites (extended stretches of repeated nucleotide motifs) which are hot-spots for replication errors (Saunders et al., 2000). High rates of nucleotide addition and deletion occur in these regions, which may offset the codon reading frame and thereby transform the downstream sequence into non-sense or back into sense. The

rates of phase shifting vary among genes and among strains of *N. meningitidis*. For example, switching rates in receptors responsible for iron uptake have been measured to range from 2 X 10^{-2} to 7 X 10^{-6} per c.f.u. (colony forming unit) (Richardson & Stojiljkovic, 1999), which differ from the rates of phase variation found in other genes (Murphy et al., 1989; Jonnson et al., 1991; Hammerschmidt et al., 1996). Analysis of meningococcal strains from worldwide epidemics revealed frequent elevations in rates of phase shifting. This is consistent with the view that the demands of between-host colonization may favour high rates of phase shifting of genes associated with colonization. Approximately half of 100 serogroup A isolates sampled from the epidemics were found to be fast shifters (rates greater than 5 X 10^{-5} per c.f.u.), and estimates of this ratio continue to increase as more strains are analysed. Furthemore, the prevalence of fast shifters in this set of strains increases as the epidemic progresses in time (Richardson et al., 2002). One possible explanation is that, as the epidemic progresses, an increasing fraction of the population has acquired one or more antigenic variants of *N. meningitidis*. Subsequent infections of these individuals require changes in the antigenic state of the bacterium, such as those that could be generated by phase shifting. Probably, the rapid phase shifting is evolved and maintained by selection operating at an epidemiological level, as a mechanism to facilitate colonization and infectious transmission to new immunologically diverse hosts, increasing the likelihood that colonizing populations of *N. meningitidis* will cause invasive disease.

Presumably, the virulence of *N. meningitidis* does not confer any long-term advantage to the bacteria in its ability to spread through a community of hosts (and may even be detrimental), but rather is an inadvertent consequence of mutation and selection within individual hosts. Levin and Bull have called this phenomenon the “short-sighted” evolution of virulence (Levin & Bull, 1994; Taha et al., 2002).

DNA RECOMBINATION AND REPAIR IN PROKARYOTES

Most of our understanding about the processes of DNA recombination and repair in prokaryotes comes from studies on model organism *Escherichia coli*. Decades of biochemical and genetic investigation have produced a detailed knowledge of the molecular mechanisms underlying these basic processes of DNA metabolism, providing paradigms and milestones (Modrich, 1991; Kuzminov, 1999). In *N. meningitidis*, microorganism that is considered as a paradigm of genetic variation, several molecular processes are similar to *E. coli*, others appear to be divergent (Kline et al., 2003; Davidsen & Tonjum, 2006).

The genetic basis for this variation depends on the evolution of iterative DNA motifs, especially homo-polymeric tracts, to effect reversible, high frequency molecular switching through slippage-like mechanisms. The variation in number of homo-polymeric repeats affects gene expression by transcriptional or translational mechanisms (Bayliss, et al., 2004). Although the mutation rate at homo-polymeric repeats is high in all strains, several strains isolated from patients may be hyper-mutable at these loci due in part to genetic defects in the DNA mismatch repair (MMR) process. Epidemic serogroup A isolates have elevated missense mutation rates and phase variation frequencies, which were caused by defects in MMR proteins, MutL and MutS in about 40% of the strains (Richardson et al., 2002).

DNA mismatch repair, one of several DNA repair pathways conserved from bacteria to humans, plays a critical role as a guardian of the genome in virtually all cells. It contributes about 1000-fold to the overall fidelity of replication and targets mispaired bases that arise through replication errors, during homologous recombination, and as a result of DNA damage.

In *E. coli* MMR recognizes base–base mismatches except C/C or small insertion/deletion nucleotide loops (IDLs) up to four nucleotides in length, which are intermediates in frameshift mutation, and probably also to sites of DNA damage (Mello et al., 1996). Mismatch recognition by MutS (homodimeric ATPase), in the presence of ATP, drives MutL (member of

the GHKL superfamily of ATPase) recruitment and subsequent activation of MutH, an endonuclease that recognizes hemi-methylated d(GATC) sequences, and cleaves the un-methylated sites spanning the mismatch. The nick serves as a point of entry for Ssb (single-stranded DNA binding protein) and halicase II (for example UvrD, MutU), whose loading at the nick is facilitated via protein-protein interactions with MutL. d(GATC) adenine methylase (Dam) plays a key role in this process, as during DNA replication only the parental strand is fully methylated and methylation of daughter strand is not immediate, allowing directionality to MMR (Modrich, 1989; 1991). Excision of the newly synthesized strand between the nick and the mismatch is carried out by one of four single-strand exonucleases: 3'-5' exonucleases ExoI or ExoX, or 5'-3' exonucleases RecJ or Exo VII (Viswanathan & Lovett, 1999; Burdett et al., 2001). DNA polymerase III, Ssb, and DNA ligase carry out repair synthesis (Fig.5).

There are several important aspects that differentiate MMR in *E. coli* and *N. meningitidis* (Davidsen & Tonjum, 2006). These include: (i) lack of MutH endonuclease in all meningococci (Richardson et al., 2002); (ii) absence of Dam methylase in several meningococcal virulent lineages (Bucci et al., 1999; Jolley et al., 2004); (iii) lower spontaneous mutation rates in meningococcal MutS- or MutL-defective strains compared with MutY-defective mutants (Richardson et al., 2001; 2002). In contrast, in *E. coli* MutS- or MutL-defective mutants exhibit stronger mutator phenotypes than MutY-defective strains (Michaels et al., 1992). Clearly, mechanisms other than MMR are implicated in meningococcus mutator phenotypes, as alterations of the DNA glycosylase MutY involved in the base excision repair pathway and in the protection against oxidative stress (Davidsen et al., 2005). Tuning down the level of activity of the MMR system, also in MMR-proficient strains, may be associated to natural competence of meningococci for transformation. The importance of natural transformation among mucosal pathogens is well established: it promotes horizontal exchange

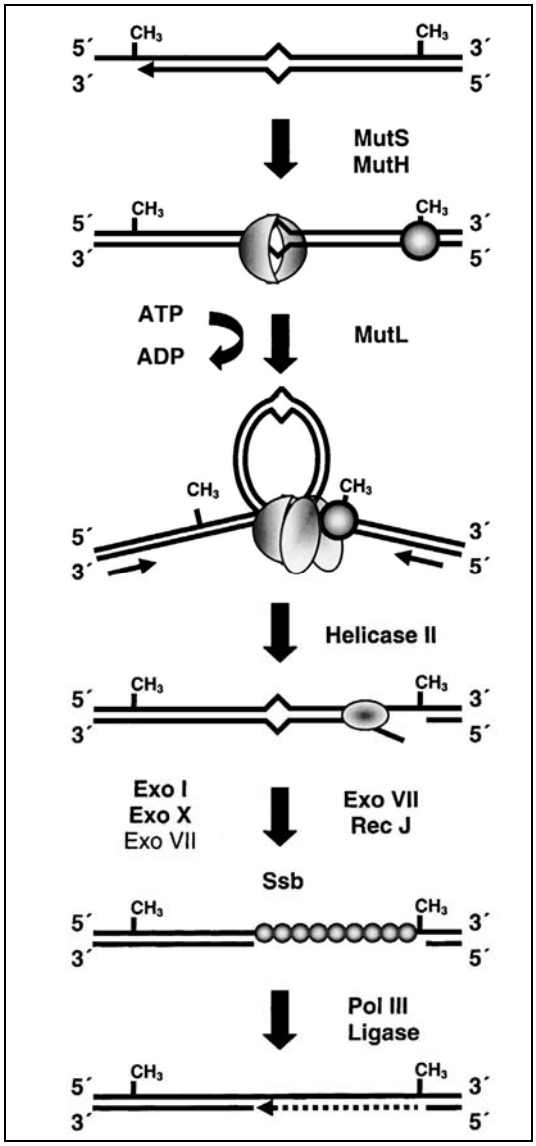

Figure 5. The methyl-directed MMR system of *E. coli*

Mismatches that arise during replication are recognized by dimeric MutS protein and hemimethylated *dam* sites are bound by MutH. Binding of ATP mediates formation of the ternary complex heteroduplex DNA-MutS-MutL, and ATP hydrolysis activation of MutH, which incises the newly synthesized and transiently unmethylated strand. Further steps include DNA unwinding by helicase II from the site of the nick followed by degradation of the single strand by one of several partially redundant 5'-3' exonucleases or 3'-5' exonucleases. The single-stranded template strand is protected by Ssb. Resynthesis is performed by polymerase III and the remaining nick is sealed by ligase.

of genetic material among *Neisseria*, responsible for the mosaic nature of virulence factors and housekeeping genes (Alexander et al., 2004; Davidsen & Tonjum, 2006).

Mismatch repair also is known to be a barrier to interspecies recombination. Indeed, MutS, MutL and MutH play a role in blocking DNA recombination between partially divergent sequences, known as homeologous recombination. For example, interspecies gene transfer between *Salmonella* and *E. coli* is normally repressed but becomes unrestrained when mismatch repair proteins, MutS and MutL in particular, are absent. Prevention of recombination between divergent sequences ensures the stability of the genome and individual species (Matic et al., 1996; Stambuk & Radman, 199; Harfe & Jinks-Robertson, 2000).

In *N. meningitidis*, the antigenic variation of several virulence determinants allows the optimization of adhesion and invasion processes by finely tuning intermolecular interactions with the available host receptors, and, at a later stage, to escape the immune response (Robertson & Meyer, 1992). Antigenic variation involves both homologous recombination activities and specific factors (Wainwright et al., 1994; 1997).

Homologous recombination is described in detail in *E. coli*, where genetic studies are delineated two recombinational pathways; the primary, RecBCD pathway, serving the needs of "sexsual" recombination, and the secondary, RecF pathway, kicking in when the primary pathway is inactive and moonlighting at "postreplication repair" of daughter strand gaps (Clark, 1973; 1988; Clark & Sandler, 1994). Biochemical characterization of recombinational activities suggested that their primary role is in DNA repair (Cox, 1991; 1993).

Genes from both of these pathways have been found in *Neisseria* and have been shown to have a role in DNA recombination and repair mechanisms. RecA and the RecF-like pathway (RecO-, RecQ- and RecJ-dependent) of recombination, as well as RecX and Rep helicase, are essential for pilin antigenic variation (Koomey et al., 1987; Mehr & Seifert, 1998). In contrast,

recombination during transformation partially requires the activity of the RecBCD pathway in addition to RecN, RecX and Rep (Salvatore et al., 2002; Kline & Seifert, 2005).

Important aspects that differentiate recombination pathways in *E. coli* and *N. meningitidis* are: (i) lack of an SOS response in meningococci; (ii) absence of a RecF homologue, although RecF family members do exist in this species (Kline & Seifert, 2005; Davidsen & Tonjum, 2006).

Interestingly, there is recent evidence that antigenic variation rates may differ in various clinical isolates as a consequence of genetic defects in DNA recombination pathways (Bucci et al., 1999; Richardson & Stojiljkovic, 2001; Salvatore et al., 2002). Serogroup B and serogroup C meningococci belonging to the ET-37 lineage harbouring the non-functional $recB^{ET\text{-}37}$ allele were defective both in repair of DNA lesions induced by UV treatment, and, partially, in recombination mediated transformation. The $recB^{ET\text{-}37}$ allele considerably increased the frequency of antigenic variation at the pilin loci (Salvatore et al., 2002), leading to the hypothesis that the RecBCD nuclease may compete with the RecF-like pathway for recombination intermediates of pilin gene conversion (Salvatore et al., 2002; Kline et al., 2003).

AIM OF THE STUDY

Reports from recent studies suggest that genetic lesions in DNA repair pathways may occur in natural bacterial populations. The occurrence of defective alleles of DNA repair and recombination genes is the rule, not an exception, in microorganisms. These alleles, or possibly their combination, play an important role in the long-term evolution of pathogenic traits. They affect the general mutability and/or the frequency of genetic exchanges. They also influence by more specialized mechanisms of genetic (phase and antigenic) variation short-term adaptation, even within a single infection cycle, of pathogenic microorganisms to their specific hosts. The cost of a reduced individual fitness, which is invariably associated to genetic lesions in DNA repair and recombination systems, is balanced by these extra benefits.

In this study we discovered a moderate mutator phenotype in several serogroup B invasive strains of *N. meningitidis*, phylogenetically related to the lineage III. The lineage III strains made their appearance in 1980, starting homogeneously with regard to both genotype (ET-24; ST-44) and phenotype (B:4:P1.4). After 1984, other clones appeared in the lineage, and the various clones acquired other serotypes (serotypes 14 and 15) and subtypes (P1.2, P1.7, and P1.12), indicating frequent exchange of genetic material between clones. In this study the mutator phenotype in the lineage III strains was associated to the presence of defective *mutL* or *mutS* alleles that were genetically characterized. In addition, we have found a novel link between the mismatch repair and the RecBCD recombination pathways.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Meningococcal invasive strains used in this study are listed in Table 2. Serotypes, sero-subtypes and assignment of the strains to hypervirulent lineages are shown in the table. The eight meningococcal strains phylogenetically related to the lineage III (ET-24) were isolated during outbreaks of epidemic disease, which have occurred in different places of Italy and France during the last 20 years. BL892 conserved the same serotype and subtype (B:4:P1.4) as the original lineage III strains isolated in 1980 (Scholten et al., 1994). The other strains acquired other serotypes and subtypes (serotype 1 in BL942; subtypes P1.1 in BL951, and P1.13 in an epidemic clone including five strains, BL851, BL859, BS843, BS845 and BS849) or were not serotypable or subtypable. The eight strains were indistinguishable by comparing restriction fragment length polymorphysm (RFLP) patterns in eight housekeeping genes, and, therefore, they were all assigned to the same genetic lineage (Salvatore et al., 2001).

All meningococcal strains were cultured on chocolate agar (Becton-Dickinson) or on GC-agar or broth supplemented with 1% (v/v) Polyvitox (Bio-Merieux) at 37 °C in 5% CO2.

E. coli strain DH5α was used in cloning procedures. Luria-Bertani (LB) was used as a growth medium. When requested, LB was supplemented with ampicillin (100 μg ml^{-1}).

DNA procedures

High molecular weight genomic DNA from the different *N. meningitidis* strains was prepared as previously described (Bucci et al., 1999). DNA fragments were isolated through acrylamide slab gels and recovered by electroelution as described (Sambrook et al., 2001). Coding regions

of the genes *mutS* and *mutL* were amplified from chromosomal DNA by using PCR with the primer pairs mutS1–mutS2 and mutL1–mutL2 (Table 1), respectively. The oligonucleotide pair mutL1Sal5/37–mutL2Bam (Table 1) was used to provide the *mutL*-coding sequences with flanking *Sal*I and *Bam*HI restriction sites for cloning strategies. The amplification reactions consisted of 30 cycles including 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C and 1–2 min of extension at 72 °C. They were carried out in a Perkin-Elmer Cetus DNA Thermal Cycler 480. Labeling of the DNA fragments to be used as probes in Southern blot experiments was performed using standard protocols (Sambrook et al., 2001). The nucleotide sequences of the amplified coding region of *mutS* and *mutL* were determined by the dideoxy chain termination procedure using, as primers, the following oligonuleotides (Table 1): mutS1, mutS2, mutS3, mutS4, mutS5, mutS6, mutS8, mutS9, mutS10, mutS11, mutSIS1 and mutSIS2 (for *mutS*); mutL1, mutL2, mutL3, mutL4 and mutL5 (for *mutL*). DNA sequencing was performed as a service by CEINGE Biotecnologie Avanzate s.c.a.r.l., Napoli (Italy). Deduced amino acid sequence similarity searching was performed with the BLAST program using the conserved domain database (CDD) available at the National Center for Biotechnology Information (NCBI). Prediction of coiled coils from protein sequences was obtained using the Lupas algorithm (Lupas et al., 1991) available at the Pôle Bioinformatique Lyonnais (PBIL).

Plasmids, cloning procedures and transformation of meningococci

A 4548 bp-long *Sal*I DNA fragment derived from pNMRecBET-37 (Salvatore et al., 2002), spanning the entire coding region of $recB^{ET\text{-}37}$, was used to transform the UV-resistant strain BL892. UV-sensitive transformants were isolated with a frequency of about 0.5%. Replacement of $recB^{ET\text{-}37}$ for the wild-type *recB* allele was verified by Southern blot analysis as previously described (Salvatore et al., 2002). To construct pDE-MutLET-5, the DNA

Table 1. Oligonucleotides used in this study

Name	Sequence
mutL1	5′- GGTATTTTCCAAACGGGATAAACGGC-3′
mutL2	5′-TCAAAGTCAGTTTGACCCAAGTCGG-3′
mutL3	5′-CAATACTGCTTCGTCAACCATCG-3′
mutL4	5′-AAGCGGATTTCGGTTTTGGTCG-3′
mutL5	5′-TCGTGATGTTTCTGTCGGTGCG-3′
mutL1Sal5/37	5′-GGAAGCGTCGACTATGTCCCGAATCGCCGCCCTGCC-3′
mutL2Bam	5′-TATTCT GGATCCTTTCGGCTTACTGTCCGCGCAAG-3′
mutS1	5′-AAATCCGCCGTTTCCCCAATGATGC-3′
mutS2	5′-TACAGTTCTGACAATGCTTCGCGC-3′
mutS3	5′-CTCGAAACCGACAGCCAATATATCG-3′
mutS4	5′-CAAAACCAAGCTGACAACTGTGG-3′
mutS5	5′-TGGATTTGGAAGCCAAGGAACGC-3′
mutS6	5′-TAGGTCAGCTCGAAATAGTGGG-3′
mutS8	5′-TAACACCCGATGCCGTCTGAAGTTG-3′
mutS9	5′-AATATGGGCGGCAAATCCACCTAC-3′
mutS10	5′-GGTTGGATTTGGTGCAGGAAAACG-3′
mutS11	5′-AAACGGCACGCCTGCCATTTTGAC-3′
mutSIS1	5′-TTGCGCACTCACTTTAATCAGTCCG-3′
mutSIS2	5′-CGAATTGCTGGAACTGATCAACCG-3′
recB1Hind	5′-GCAACAAGCTTCCGGCTGCAACTGGATTTAATCG-3′
recB2Hind	5′-GGCGGCAAGCTTTTCGAGCAGTTGGAAATAATTGG-3′

corresponding to the entire *mutL*-coding region was amplified from the genome of MC58 (ET-5) using primers mutL1Sal 5/37 and mutL2Bam.

The *Sal*I–*Bam*HI-restricted 1991 bp PCR product was cloned into the *Sal*I–*Bam*HI-restricted pDEX vector plasmid (Pagliarulo et al., 2004). To obtain pDEΔ-RecB, the central coding region of *recB* from MC58 was amplified using the primer pair recB1Hind/recB2Hind. Then, the *Hin*dIII-restricted 1101 bp PCR product was cloned into *Hin*dIII-restricted pDEX.

Transformation of meningococci was performed as previously described (Salvatore et al., 2002) by using 0.1–1 μg of plasmids or DNA fragments. Transformants were selected on GC-agar medium containing erythromycin (7 μg ml^{-1}) when requested.

UV survival assays

Meningococcal cells were grown to late logarithmic phase in GC medium supplemented with Polyvitox. When the cultures reached an optical density at 550nm (OD_{550nm}) of 1.0, 10 μl of serial dilutions were spotted onto Petri dishes containing GC-agar plus Polyvitox supplement. Irradiation was achieved by opening the plates under a SpectrolineR model ENF-260C/F UV(254) germicidal lamp (Spectronics Corporation, Westbury, NY, USA) at a distance of 15 cm for different times, in the absence of daylight illumination. As a control, dilutions from the same cultures were spotted onto non-irradiated plates. UV fluences were determined as described (Salvatore et al., 2002). Exposure to the germicidal lamp never resulted in an increase of temperature of the GC-agar medium more than 0.1 °C, as measured by platinum probe (Pt-RTD 100Ω) that was placed at the surface of the medium.

Determination of spontaneous mutation frequencies

In these assays strains were grown in GC broth supplemented with 1% (v/v) Polyvitox (Bio-Merieux) at 37 °C with shacking to late logarithmic phase (1.0 OD_{550nm}), starting from single colonies grown over-night in GC-agar with 1% (v/v) Polyvitox at 37 °C in 5% CO2. For each strain, a total of 300 colonies were tested in 10 distinct experiments (30 colonies for each strain in each experiment). After growth, 1 ml cultures (about 1×10^9 colony forming units [cfu]) were plated onto selective GC-agar containing 36 μg ml^{-1} rifampicin (Sigma–Aldrich s.r.l.). Serial dilutions were treated in parallel and plated on GC-agar to determine the viable cfu. Rifampicin-resistant colonies were counted after 24 h incubation at 37 ◦C in 5% CO2. Mutation frequencies were calculated in each set of experiment by using the method of the median (Lea & Coulson, 1949) that was proved to be accurate for our purposes by preliminary experiments (Rosche & Foster, 2000). Values in Tables 2, 4 and 5 represent means of mutation frequencies (medians) to rifampicin-resistance obtained in each set of experiments. For statistical analysis a standard *t*-test (Zar, 1984) was used. Fluctuation is usual with the mutation rates seen for rifampicin-resistance so that only differences of a factor 2 or more were considered significant (Glickman & Radman, 1980). By using lower rifampicin concentration (9 μg ml^{-1}) we did not observe substantial change in the relative mutability of the strains.

RESULTS

Mutation frequencies to rifampicin-resistance in lineage III meningococcal strains

In Table 2 spontaneous mutation frequencies to rifampicin resistance were determined in the lineage III isolates and in several meningococcal strains belonging to the ET-5 and to the ET-37 (serogroup B and C) hypervirulent lineages as detailed in Materials and Methods. The lineage III strains exhibited different mutation frequencies. When compared to the ET-5 strain MC58, the lineage III strains BL942 and BL892 exhibited, respectively, about 7.8- and 2.4-fold higher mutation frequencies. A moderate mutator phenotype was also present in BL951 (1.9-fold higher mutation frequencies than in MC58), but not in the epidemic clone including strains BL851, BL859, BS843, BS845 and BS849. In this assay, all tested ET-5 strains (BL899, BL937, BF23, BZ169, H44/76 and MC58) and most ET-37 strains (BF2, BF40 and NGP165) gave similar values, with the exclusion of the ET-37 strain 93/4286 that showed a clear mutator phenotype (7.7-fold higher mutation frequencies than in MC58).

Table 2. Mutability to rifampicin-resistance of meningococcal clinical isolates

Strain	Serogroup and/or serotype[a]	Lineage[b]	Clinical specimen[c]	Mutation frequency[d] per 10^9 cells, median n°	Source[e]
BL851	B:4:P1.13	ET-24	CSF	2.81	i.
BL859	B:4:P1.13	ET-24	CSF	3.12	i.
BS843	B:4:P1.13	ET-24	BL	2.79	i.
BS845	B:4:P1.13	ET-24	BL	2.68	i.
BS849	B:4:P1.13	ET-24	BL	3.20	i.
BL892	B:4:P1.4	ET-24	CSF	5.67	ii.
BL942	B:1:NST	ET-24	CSF	18.75	ii.
BL951	B:NT:P1.1	ET-24	CSF	4.46	ii.
BL899	B:4:P1.2,5	ET-5	CSF	1.93	ii.
BL937	B:4:NST	ET-5	CSF	1.85	ii.
BF23	B	ET-5	NP	2.60	iii.
BZ169	B	ET-5	CSF/BL	2.47	iv.
H44/76	B	ET-5	CSF/BL	2.53	iv.
MC58	B	ET-5	CSF/BL	2.39	iv.
BF2	B	ET-37	NP	2.68	v.
BF40	B	ET-37	NP	2.52	iii.
93/4286	C	ET-37	CSF/BL	18.41	iv.
NGP165	B	ET-37	CSF/BL	1.92	iv.

[a]NT, not serotypable; NST, not sub-serotypable.
[b]The assignment of the isolates to hypervirulent lineages has been determined as previously described (Salvatore et al., 2001). ET-24 or lineage III.
[c]NP, nasopharynx; CSF, cerebro-spinal fluid; BL, blood.
[d]Data were obtained by 10 independent experiments. In each experiment, 30 independent cultures of each strain were tested. Values represent means of mutation frequencies (medians) to rifampicin resistance obtained by mutagenicity assay in each set of experiments. For each strain these values did not differ more than 20%.
[e]i. Istituto Superiore di Sanità, Roma, Italy; ii. Institut Pasteur, Paris, France. iii. Hôpital d'Instruction des Armée, BREST NAVAL, France; iv. IRIS, Chiron S.p.A, Siena, Italy; v. Laboratory of Microbiology, Università di Napoli, Italy.

Nucleotide sequences of *mutS* in meningococcal strains

As a mutator phenotype is frequently caused in meningococci by mutations in mismatch repair genes *mutL* and *mutS* (Richardson & Stojiljkovic, 2001) the nucleotide sequence of *mutS* was determined in the lineage III strains BL892, BL951 and BL942, and in the ET-37 strain 93/4286. The alignment of the deduced amino acid sequences of the *mutS* genes from BL892 and MC58 demonstrated a high degree of similarity (>98% identity). The 18 amino acid substitutions we found in the MutS protein of BL892 affected either synonymous (9 of 18) or non-conserved residues (9 of 18) on the basis of the available consensus for the MutS family proteins (COG0249 at the NCBI data base). Similarly, the MutS protein of BL951 exhibited only 15 amino acid substitutions compared to that of MC58. Twelve out of these fifteen substitutions were also found in the MutS protein of BL892, and the remaining three substitutions affected non-conserved residues. In contrast, the *mutS* allele from BL942 was functionally inactive as a consequence of a cytosine deletion at position 235 of the coding region responsible for shifting of the reading frame (Fig. 6). The *mutS* allele from 93/4286 was subjected to a different mechanism of inactivation: insertion of an IS*1106* element (Salvatore et al., 2001), which targeted the sequence AGCCT at position 2146–2150 of the nucleotide sequence of the *mutS* coding region. This insertion was predicted to interrupt the ATPase domain of MutS (Fig. 6).

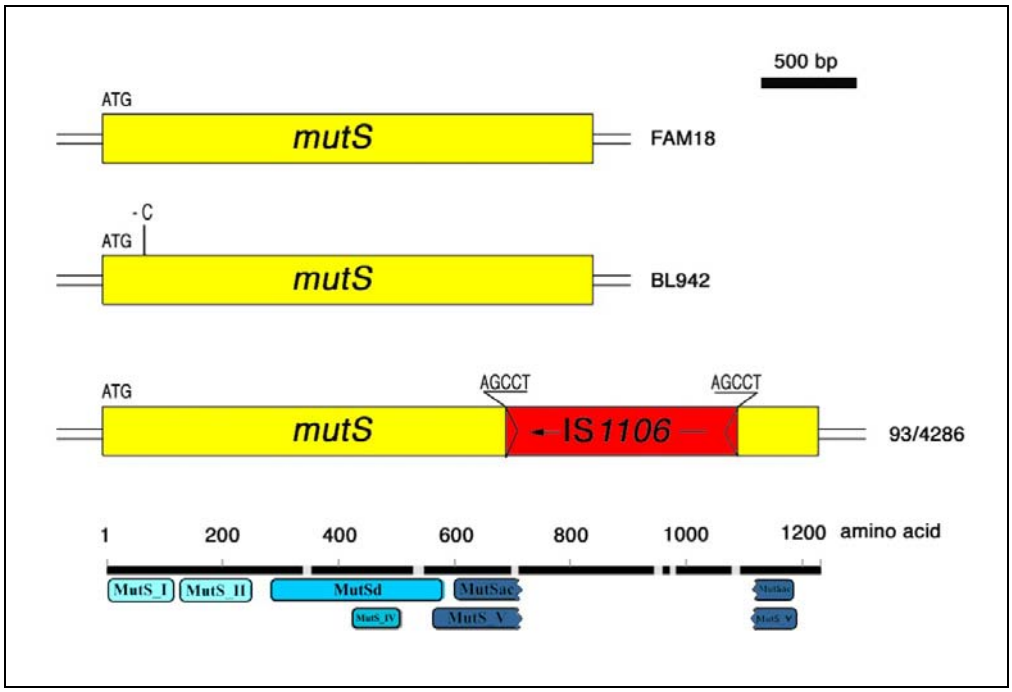

Figure 6. Insertional and frame-shift inactivation of *mutS* in meningococcal strains

The genetic map of the *mutS* gene from the ET-37 strains FAM18 and 93/4286, and the lineage III strain BL942 is depicted in the upper part of the figure. The *mutS* gene from 93/4286 is interrupted by an IS*1106* transposable element, while that from BL942 contains a frame-shift mutation (a deletion of one cytosine residue). The repeated sequences at the level of the insertion site are indicated. The functional domains of MutS protein are drawn below the genetic map. MutS I: domain corresponding to globular domain I in *Thermus aquaticus* MutS (Obmolova et al., 2000); MutS II: domain corresponding to globular RNAse-H-like domain II in *T. aquaticus* MutS; MutS IV: domain corresponding to globular domain IV in *T. aquaticus* MutS, which is involved in DNA binding; MutS V: domain corresponding to globular domain V in *T. aquaticus* MutS, which contains a Walker A motif, and is structurally similar to the ATPase domain of ABC transporters; MutSd: DNA-binding domain of MutS; MutSAc: ATPase domain of MutS.

Mutations of meningococcal *mutL* allele in different virulent strains

The alignment of the deduced amino acid sequence of the *mutL* genes from the lineage III strains with the available sequences from MC58 (ET-5), FAM18 (ET-37) and Z2491 (IV-I) revealed the presence of multiple amino acid substitutions differently distributed among the strains. Most of these substitutions coincided with those previously identified by Richardson and Stojiljkovic (Richardson & Stojiljkovic, 2001) in a meningococcal mutator *mutL* allele (Table 3). In particular, the highest number of amino acid substitutions (21 out of the 23 typical of the mutator allele) was detected in the *mutL* allele from BL942, the strain that also carried a functionally inactive *mutS* allele, and exhibited the strongest mutator phenotype (Table 2). In the *mutL* allele from BL892 ($mutL^{BL892}$) the number of amino acid substitutions was lower (17) and even lower in the *mutL* alleles from BL851 and BL951 (11). The *mutL* alleles from MC58 (ET-5), Z2491 (IV-I) and FAM18 (ET-37) exhibited, respectively, 11, 12 and only a single amino acid substitution. This result may be of relevance because the ET-37 strains, except 93/4286, seem to be generally characterized by lower spontaneous mutability (Table 2) and lower ability to be transformed by exogenous DNA (Salvatore et al., 2002). The *mutL* allele from the mutator strain 93/4286 was almost identical to that from FAM18 (data not shown).

Table 3. Substitutions in the deduced amino acid sequences of MutL proteins[a]

Strain	G62S	A165N	D226G	D331N	S337G	T350V	P351S	N365D	R366S	N369D	S376P	H379R
FAM18	–	–	–	–	–	–	–	+	–	–	–	–
MC58	–	+	+	+	+	+	+	+	+	–	–	–
Z2491	+	+	+	–	–	–	–	–	–	+	+	+
BL892	+	+	+	–	–	–	–	+	+	+	+	+
BL942	+	+	+	+	+	+	+	+	+	+	+	+
BL851	+	–	+	–	+	–	–	–	–	+	+	+
BL951	+	–	+	–	+	–	–	–	–	+	+	+

Strain	S395A	Y397R	R402H	F433L	Q449K	N513K	V523S	A562V	N580G	F582L	H597R
FAM18	–	–	–	–	–	–	–	–	–	–	–
MC58	–	–	–	–	–	–	–	+	–	+	+
Z2491	–	–	–	–	+	+[b]	+[c]	+	+	+	–
BL892	+	+	+	+	–	+	–	+	+[d]	+	+
BL942	+	+	+	–	–	+	+	+	+	+	+
BL851	–	–	–	–	–	+[b]	–	+	+	+	+
BL951	–	–	–	–	–	+[b]	–	+	+	+	+

[a]Only substitutions that are typical of *mutL* mutator alleles (Richardson and Stojiljkovic, 2001) are indicated. [b]N513R; [c]V523I; [d]N580S.

mutL allelic replacement in the lineage III strain BL892

To verify the causal association between genotype and mutator phenotype, the *mutL* gene in the BL892 chromosome was replaced by the homologous gene from MC58 using the linearized plasmid pDE-MutL^{ET-5} (Fig. 7A). The allelic replacement by transformation and double cross-over was verified by Southern blot by screening 96 recombinant strains (Fig. 7B). In only one strain, BL892 mutL^{ET-5}, *mutL* was almost entirely replaced. In this strain, as a result of the recombination event, two *Hin*fI DNA fragments of the expected sizes (503 and 630 bp) were detected by *mutL*-specific probe (Fig. 7B, lane 3), in place of the about 2500 bp-long *Hinf*I fragment of the parental strain BL892 (Fig. 7B, lane 1).

This result was confirmed by PCR restriction analysis (Fig. 7C) using a primer pair designed to amplify the entire *mutL*-coding region (Fig. 7A) followed by *Hin*fI digestion. The restriction pattern of BL892 mutL^{ET-5} (Fig. 7C, lane 4) was almost identical to that of MC58 (Fig. 7C, lane 2) with exception of the more distal 154 bp DNA fragment, specific of MC58, at the recombination site (Fig. 7A). The allelic replacement suppressed the mutator phenotype of BL892 resulting in mutator levels comparable to those observed in MC58 (Table 4). This finding demonstrated that the enhanced mutability of BL892 strain was the result of non- or less-functional *mutL* allele.

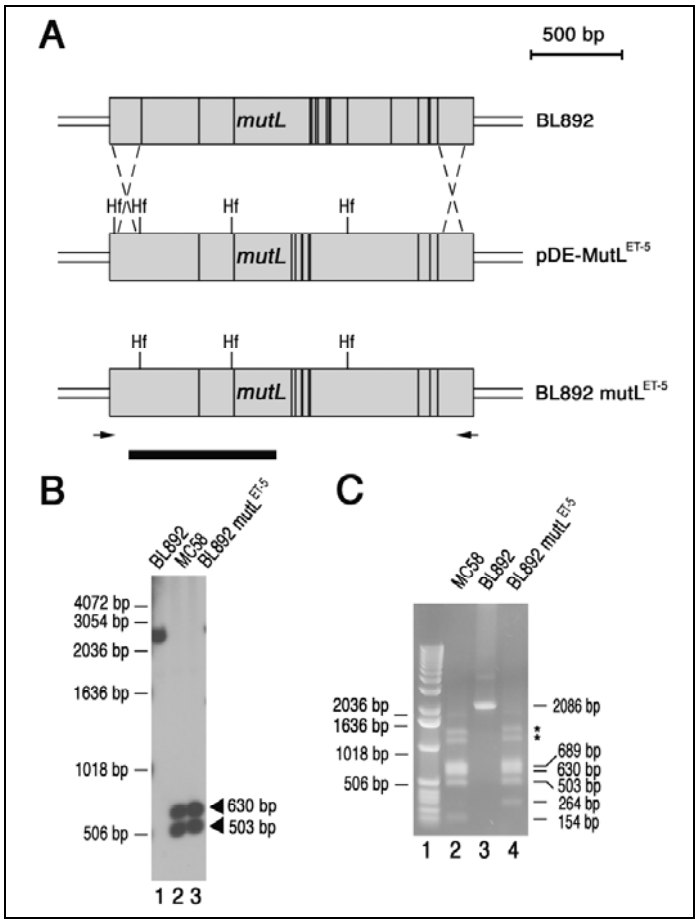

Figure 7. *mutL* allelic replacement in BL892

(A) Experimental design for *mutL* allelic replacement in BL892 generating BL892 mutL^{ET-5} by double cross-over using linearized pDE-MutL^{ET-5}. Location of *Hin*fI (Hf) restriction sites and distribution of missenses (bar) are reported. Positions of the DNA probe (black rectangle) used in Southern blot experiment (B) and of the primer pair (arrows) used in PCR-restriction analysis (C) is also indicated.
(B) Southern blot analysis. Total DNAs derived from BL892 (lane 1), MC58 (lane 2) or BL892 mutL^{ET-5} (lane 3) were digested by *Hin*fI and analyzed by Southern blot using the DNA probe indicated in (A). Relative migration of DNA molecular weight ladders is reported on the left. Arrowheads on the right indicate MC58-specific fragments.
(C) PCR-restriction analysis. Total DNAs derived from MC58 (lane 2), BL892 (lane 3) or BL892 mutL^{ET-5} (lane 4) were used as templates in PCR reaction with the primer pair (mutL1 and mutL2Bam) above indicated (A). The PCR products were purified and digested by *Hin*fI and analyzed by agarose gel. In lane 1 DNA molecular weight ladders were run in parallel, and their relative migration is reported on the left. The sizes of MC58- and BL892 mutL^{ET-5}-specific fragments are indicated on the right.

Table 4. Effects of *mutL* allelic replacement or integration of plasmid pDE-MutL^{ET-5} into the chromosome of BL892 on mutability to rifampicin-resistance

Strain	Spontaneous mutation frequency[a] per 10^9 cells, median n°
BL892	5.67
BL892 mutL^{ET-5}	2.45
BL892ΩmutL^{ET-5}-2	2.49
BL892ΩmutL^{ET-5}-6	5.65
BL892ΩmutL^{ET-5}-13	5.50
BL892ΩmutL^{ET-5}-14	2.52
BL892ΩmutL^{ET-5}-15	2.66

[a]Mutation frequencies to rifampicin resistance were determined as described in footnote to Table 2.

Cointegration assay in the *mutL* locus of BL892

The result of allelic replacement was further supported by mutagenicity assays in cointegrant strains. To this purpose, plasmid pDE-MutL^{ET-5}, harbouring a promoter-less *mutL* allele from MC58 was used to generate cointegrates with the chromosome of BL892 (Fig. 8A). Different *mutL* chimeric genes were generated as a result of integration of pDE-MutL^{ET-5} into the host chromosome. Regions where recombination occurred were mapped in the different cointegrant strains by PCR-restriction analysis (Fig. 8B). This analysis demonstrated that recombination occurred very close to the 5'-end of *mutL* gene (within the 154 bp *Hin*fI fragment) in BL892ΩmutL^{ET-5}-2 (Fig. 8A and B, lane 6), within the 5'-end-proximal 503 bp *Hin*fI fragment in both BL892ΩmutL^{ET-5}-14 and BL892ΩmutL^{ET-5}-15 (Fig. 8A and B, lanes 4 and 5, respectively), within the central 630 bp *Hin*fI fragment in BL892ΩmutL^{ET-5}-13 (Fig. 8A and data not shown), and within the 3'-end-proximal 689 bp fragment in BL892ΩmutL^{ET-5}-6 (Fig. 8A and data not shown). Mapping by PCR-restriction was confirmed by Southern blot analysis (data not shown). In strains BL892ΩmutL^{ET-5}-2, BL892ΩmutL^{ET-5}-14 and BL892ΩmutL^{ET-5}-15 the mutator phenotype of BL892 was suppressed as well as in BL892 mutL^{ET-5} (Table 4). In contrast, BL892ΩmutL^{ET-5}-6 and BL892ΩmutL^{ET-5}-13 exhibited the same mutability as the parental BL892 (Table 4). The result of this analysis restricted to four (S395A, Y397R, R402H and F433L) the number of amino acid substitutions eventually responsible for the mutator phenotype, the remaining being also present in non-mutator strains. As all these amino acid substitutions affected residues that are not evolutionarily conserved in Gram-negative bacteria (data not shown), the molecular basis for the biochemical defect of the *mutL* allele from BL892 was not obvious. However, *in silico* analysis of the coiled coils distribution in the MutL peptides using the Lupas algorithm (Lupas et al., 1991) predicted that the F433L substitution in BL892 had the potential to alter locally the secondary structure of the protein (Fig. 9). By using the same strategy, *mutL* allele from MC58 was used to generate cointegrates with the

chromosome of BL942. However, in this case, suppression of the mutator phenotype was never observed in any cointegrant strains (data not shown), possibly due to the presence of the out-of-frame *mutS* allele (Fig. 6).We did not extend this analysis to BL951 because of its weak mutator phenotype that was expected to give inconclusive (statistically ambiguous) results.

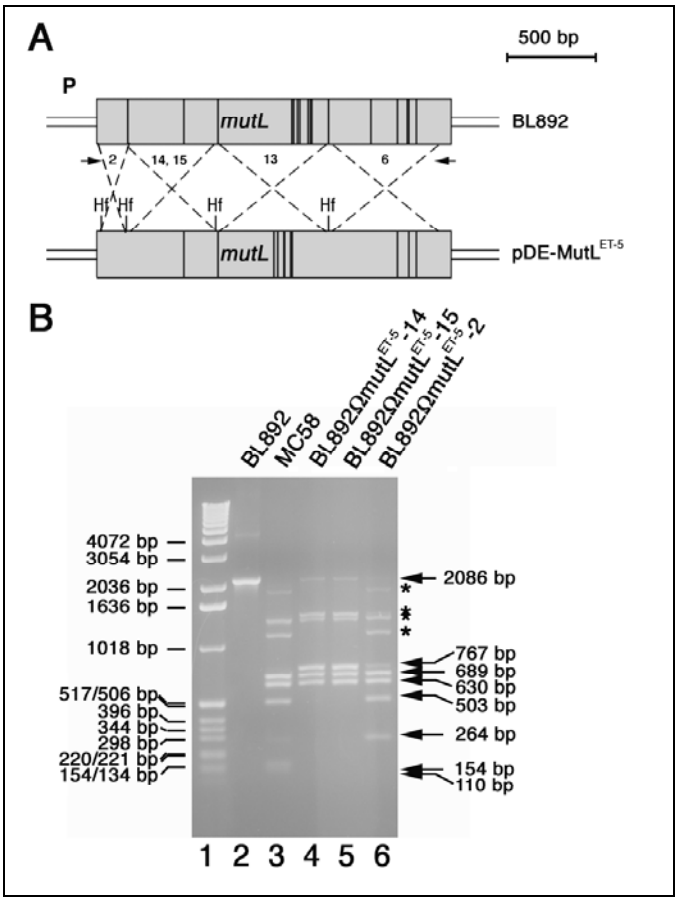

Figure 8. Generation of cointegrant strains by recombination in the *mutL* locus of BL892.

(A) Experimental design for integration of plasmid pDE-MutL^{ET-5} into the chromosome of BL892 and strategy to map the integration sites by PCR-restriction analysis. Location of the primer pair (arrows) and the *Hin*fI (Hf) restriction sites for PCR-restriction analysis (B), and the distribution of missense mutations (bar) in *mutL* alleles (see Table 3) are reported. The integration sites in strains BL892ΩmutL^{ET-5}-2 (2), BL892ΩmutL^{ET-5}-6 (6), BL892ΩmutL^{ET-5}-13 (13), BL892ΩmutL^{ET-5}-14 (14) and BL892ΩmutL^{ET-5}-15 (15) are indicated.
(B) PCR-restriction analysis. Total DNAs derived from BL892 (lane 2), MC58 (lane 3), BL892ΩmutL^{ET-5}-14 (lane 4), BL892ΩmutL^{ET-5}-15 (lane 5) and BL892ΩmutL^{ET-5}-2 (lane 6) were used as templates in PCR reaction with the primer pair (mutL1 and mutL2Bam) indicated above (A). The PCR products were purified and digested with *Hin*fI and analyzed by agarose gel. In lane 1 DNA molecular weight ladders were run in parallel, and their relative migration is reported on the left. The sizes of *mutL*-specific fragments are indicated on the right. Asterisks indicate partially digested fragments.

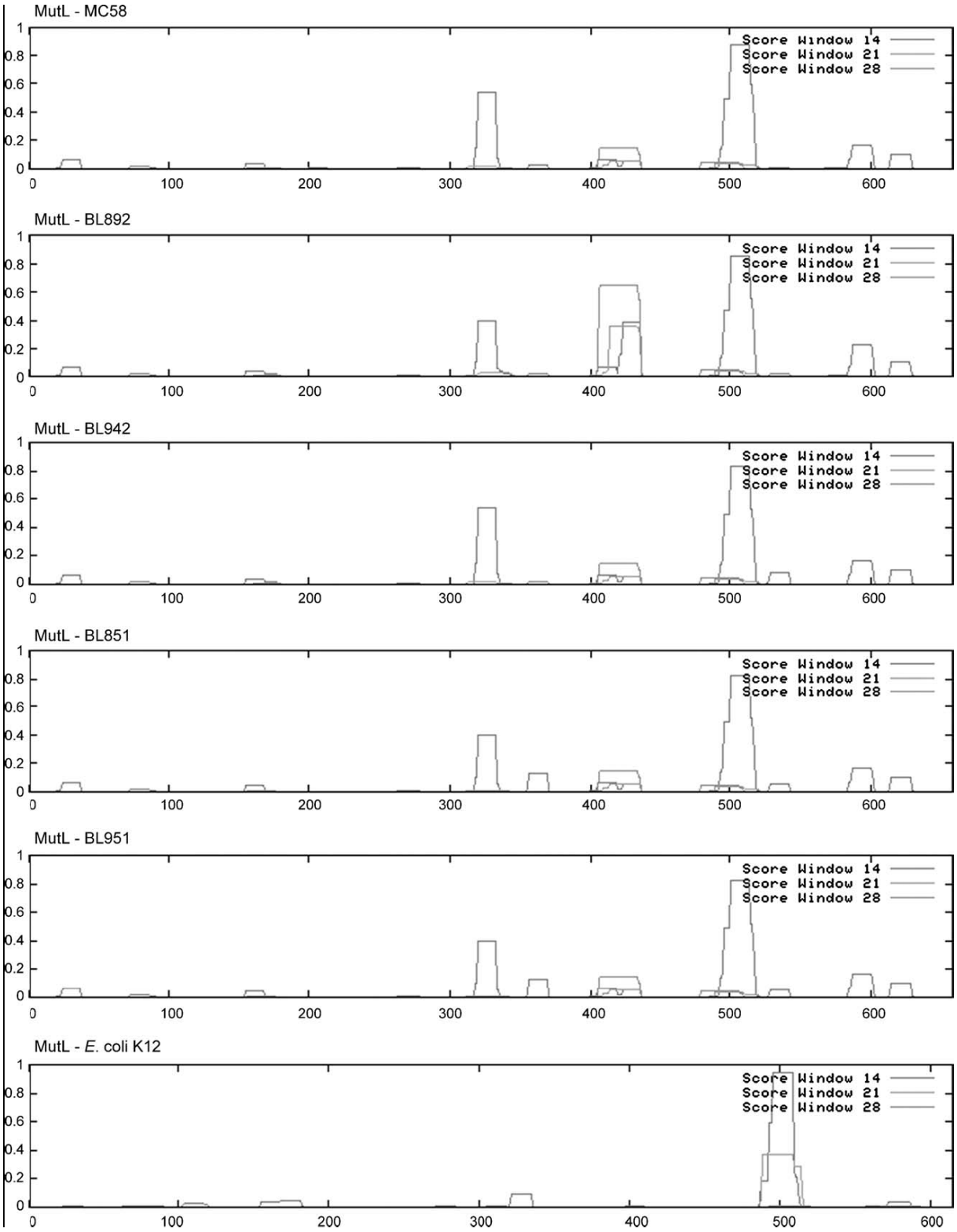

Figure 9. Coiled coil profiles of MutL deduced amino acid sequences.

Prediction of coiled coils from protein sequences was obtained using the Lupas algorithm (Lupas et al., 1991) as detailed in Materials and Metods.

Genetic interaction between *mutL*^{BL892} and *recB*$^{ET-37}$ alleles

In a previous work we demonstrated that, when introduced in the mismatch repair-proficient strain MC58, the defective *recB*$^{ET-37}$ allele did not affect the spontaneous mutability to rifampicin-resistance (Salvatore et al., 2002). We performed the same experiment with the mismatch repair-defective strain BL892. To this purpose, isogenic derivatives BL892 *recB*$^{ET-37}$ were generated by allelic replacement of the wild-type *recB* with the *recB*$^{ET-37}$ one using a previously described procedure (Salvatore et al., 2002). Transformants were selected by replica-plating selecting for increased UV-sensitivity. The results of Southern blot analysis confirmed that in all UV-sensitive clones the *recB*$^{ET-37}$ allele replaced the functional *recB* allele (data not shown). Using the same approach we attempted the same gene replacement in BL942, exhibiting a stronger mutator phenotype than BL892. However, we failed to obtain recombinants even if a large number of colonies were screened for UV-sensitivity (data not shown). In agreement with previous data (Salvatore et al., 2002) replacement of the wild-type *recB* allele with the defective *recB*$^{ET-37}$ one generated recombinant strains exhibiting moderate decrease in viability (Table 5). The recombinant strains also exhibited dramatic increase in sensitivity to hydrogen peroxide exposure. Both these effects could be detected in all strains even though they were more severe in MC58. In Table 5 the results of mutagenicity assay demonstrated that replacement of the wild-type *recB* allele with the *recB*$^{ET-37}$ one caused suppression of the mutator phenotype observed in BL892, whereas it did not affect the mutability of MC58.

Table 5. Effects of *recB* allelic replacement on meningococcal viability, mutability and sensitivity to hydrogen peroxide

Strain	Viability[a]	Viability upon hydrogen peroxide exposure[b]	Spontaneous mutation frequency[c] per 10^9 cells, median n°
MC58	**1.00**	**1.00**	**2.39**
MC58 recB^{ET-37}	**0.41**	**0.02**	**2.22**
MC58ΩrecB	**0.44**	**0.02**	**2.27**
BL892	**0.85**	**0.77**	**5.67**
BL892 recB^{ET-37}	**0.60**	**0.14**	**1.97**
BL892ΩrecB	**0.62**	**0.16**	**1.96**

[a] Viabilities of meningococcal strains were determined by counting the number of viable cells (cfu) in late exponential phase cultures ($O.D._{550\ nm}$ = 1.0) in GC medium with 1% (v/v) Polyvitox.

[b] Viabilities of meningococcal were determined after 30 min treatment of logarithmic growing bacteria ($O.D._{550\ nm}$ = 0.5) with 200 µM hydrogen peroxide.

[c] Mutation frequencies to rifampicin resistance were determined as described in the footnote to Table 2.

Insertional *recB* inactivation

To clarify whether suppression of the *mutL*-induced mutator phenotype by the defective *recB*$^{ET\text{-}37}$ was allele-specific or not, we attempted to generate null *recB* allele in *mutL*^{BL892} background. To this purpose, pDEΔ-RecB, harbouring the central coding region of *recB* from MC58, was used to transform BL892 and MC58 (Fig. 10). Transformation of MC58 resulted in insertional *recB* inactivation in almost all transformants (MC58ΩrecB). As a result of the recombination event, two *Hin*cII DNA fragments of the expected sizes (5954 and 2467 bp) were detected by *recB*-specific probe (Fig. 10B, lanes 2–8), in place of the 3293 bp-long *Hin*cII fragment of the parental strain MC58 (Fig. 10B, lane 1). By contrast, the same procedure led to *recB* inactivation only in a single BL892 transformant, BL892ΩrecB (Fig. 10B, lane 12). BL892ΩrecB and MC58ΩrecB exhibited almost the same UV-sensitivity, viability, mutability and sensitivity to hydrogen peroxide exposure as BL892 recB$^{ET\text{-}37}$ and MC58 recB$^{ET\text{-}37}$, respectively (Table 5 and data not shown). These findings suggested that *recB*$^{ET\text{-}37}$ was a null allele, and that suppression of the *mutL*-induced mutator phenotype by *recB*$^{ET\text{-}37}$ was not allele-specific.

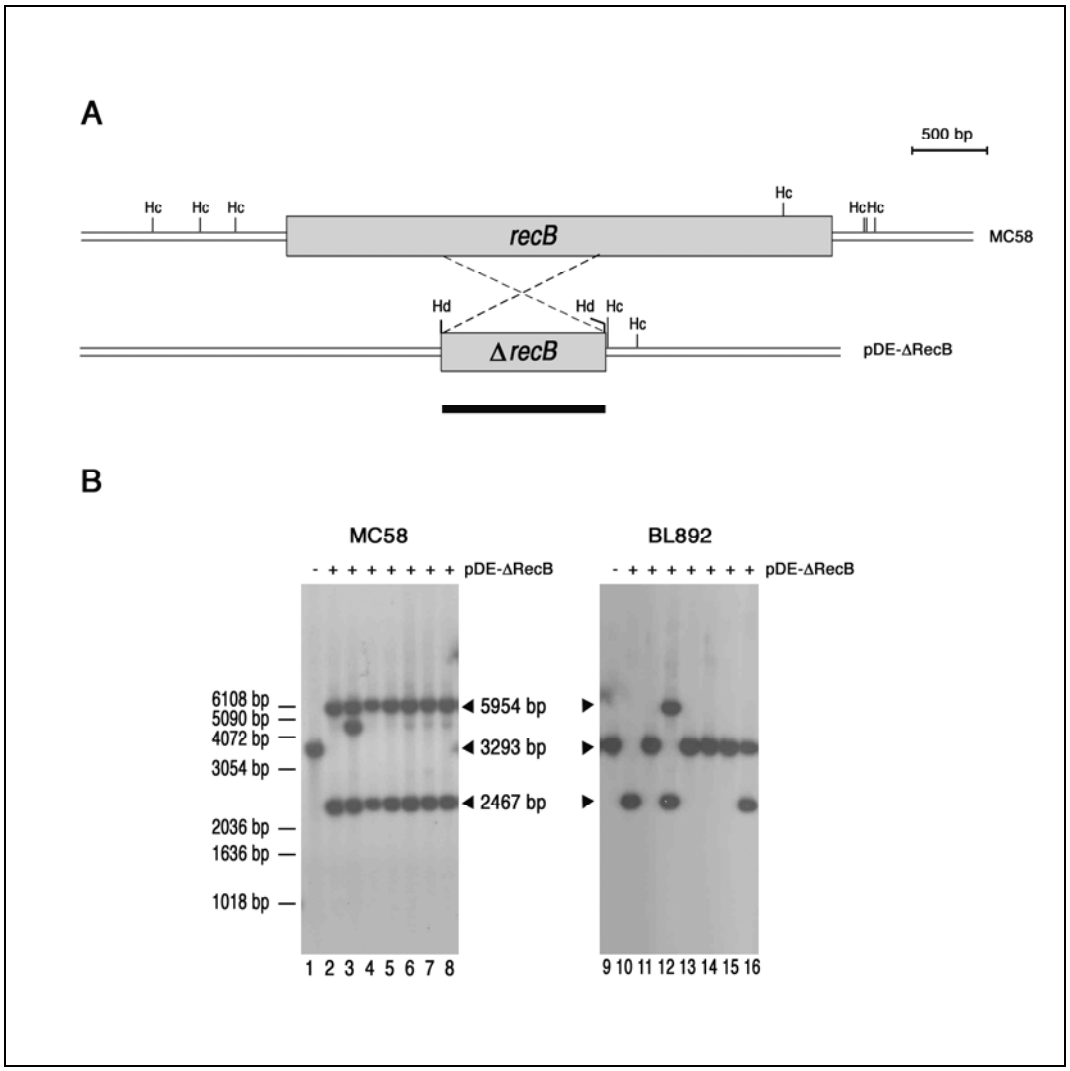

Figure 10. *recB* knock-out

(A) Experimental design for insertional *recB* inactivation by single cross-over using pDEΔ-RecB. Locations of chromosomal/plasmid *Hin*cII (Hc) and plasmid *Hin*dIII (Hd) restriction sites and of DNA probe (black rectangle) used in Southern blot experiment (B) are shown.
(B) Southern blot analysis. Total DNAs derived from MC58 (lane 1), BL892 (lane 9) and from erythromycin-resistant MC58 and BL892 derivatives transformed with pDEΔ-RecB (lanes 2-8 and 10-16, respectively) were digested with *Hin*cII and analyzed by Southern blot using the DNA probe indicated in A. Relative migration of DNA molecular weight ladders is reported on the left. Arrowheads indicate *recB*-specific fragments.

DISCUSSION

In this study we demonstrate that several invasive serogroup B meningococcal strains phylogenetically related to the lineage III exhibit a mutator phenotype (Table 2). The mutator phenotype was due to the presence of *mutL* and/or *mutS* defective mosaic alleles. The strain BL942 exhibited the strongest mutator phenotype. In this strain a loss-of-function mismatch mutation in *mutS* (Fig. 6) generated mutator levels comparable to those measured in 93/4286 (Table 2), an ET-37 strain carrying an IS*1106* insertion in the *mutS* coding region destroying the ATPase domain (Fig. 6). In addition to *mutS* mutation, BL942 also carried a *mutL* allele harbouring multiple missense mutations mostly coinciding with those previously identified in other mutator strains (Richardson & Stojiljkovic, 2001) (Table 3). Most of these amino acid substitutions were also present in BL892, a lineage III strain exhibiting a moderate phenotype (Table 2) with a functional *mutS* gene. The other lineage III strains carried mosaic *mutL* alleles and were not mutator. The results of mutagenicity assays (Table 4) after *mutL* allelic replacement (Fig. 7) demonstrated that the enhanced mutability of BL892 strain was the result of non- or less-functional *mutL* allele. The analysis of mutability of cointegrant strains harbouring chimeric *mutL* genes (Fig. 8 and Table 4) restricted to four (S395A, Y397R, R402H and F433L) (Table 3) the number of amino acid substitutions eventually responsible for the mutator phenotype. These substitutions map in the extended linker region between the N-terminal ATPase and C-terminal dimerization domains sharing no similarity between MutL homologues (Ban & Yang, 1998; Ban et al., 1999; Guarné et al., 2004). This region generates a large cavity in MutL dimers encircling DNA duplexes and tolerates sequence substitutions and deletions of one-third of its length without functional consequences (Guarné et al., 2004). In mismatch-proficient meningococcal strains the MutL linker region spans 114 residues between the amino acids 348 and 461 (332-431 in *E. coli* MutL) and it was predicted to form random

coils (Fig. 9 and data not shown) as well as in all MutL homologues (Guarné et al., 2004). *In silico* analysis of the coiled coils distribution in the MutL peptides using the Lupas algorithm (Lupas et al., 1991) predicted that the F433L substitution in BL892 had the potential to alter locally the secondary structure of the protein by enhancing dramatically the possibility to form coiled coils structure in the region spanning the amino acids 395-445 (Fig. 9). It should be interesting to test whether the extra coiled coils structure affects the biochemical activities of the MutL protein including ATPase, DNA binding, dimerization and interaction with the UvrD helicase (Ban & Yang, 1998; Ban et al., 1999; Guarné et al., 2004; Hall et al., 1998). However the evidence that deletions in the extended linker may be defective in mismatch repair *in vivo* in spite of normal ATPase and DNA binding activities (Guarné et al., 2004) recommends caution when using *in vitro* approaches.

An interesting point of this study is the evidence that the mutator phenotype of the *mutL*-defective strain BL892 may be suppressed by replacement of the functional *recB* allele with the non-functional $recB^{ET\text{-}37}$ one or by insertional inactivation of *recB* (Table 5). This result demonstrates the existence of multiple links connecting recombination and mutation in *N. meningitidis*. One of such links has been previously explored when phase variation frequencies were shown to be increased in mismatch-repair proficient meningococcal strains by transformation with heterologous DNA as a consequence of titration of some component of the mismatch repair machinery (Alexander et al., 2004).

In pathogenic *Neisseriae* the RecBCD pathway is involved in genetic transformation (Mehr & Seifert, 1998; Salvatore et al., 2002; Skaar et al., 2002; Kline et al., 2003), in pilin phase variation (Chaussee et al., 1999; Salvatore et al., 2002), and in recombinational repair (Mehr & Seifert, 1998; Salvatore et al., 2002; Skaar et al., 2002; Kline et al., 2003). There is evidence that the RecBCD pathway is mostly important for structural maintenance of chromosome replication upon fork collapse (Kuzminov, 1999). Unrepaired oxidative damage is the major

cause of replication fork collapse (Kuzminov, 1999), and this may be the reason why MC58 recB^{ET-37} and BL892 recB^{ET-37} were much more sensitive to exposure to hydrogen peroxide and were less viable than their isogenic *recB*-proficient counterparts (Table 5). Similar phenotypes were observed in *E. coli recA* or *recBC* (Ananthaswamy & Eisenstark, 1977; Carlsson & Carpenter, 1980) as well as in gonococcal *priA* mutants (Kline & Seifert, 2005).

In principle, suppression of the mutator phenotype by *recB* allelic replacement may be explained by "physical" interaction between MutL and RecB proteins along an uncharacterized mutagenic pathway. Although a similar pathway has not been found in *E. coli*, we cannot exclude that it may exist in *N. meningitidis*, a microorganism in which several DNA repair genes do not exist and the gene products of others interact differently when compared to *E. coli* (Davidsen & Tonjum, 2006). Alternatively, we hypothesize a "functional" interaction between MutL and RecB along well-established pathways. Indeed our data may suggest that in a mismatch repair-defective background mutability is mostly generated during DNA synthesis associated to RecBCD-dependent processes, such as reassembling of disintegrated replication forks. There is analogy between the mutagenesis pathway we describe and two distinct mutagenesis pathways of *E. coli* in their apparent requirement for RecBCD-dependent recombination pathway: translational stress-induced mutagenesis (TSM) (Balashov & Humayun, 2002; Mamun et al., 2002) and stationary-phase (adaptive) mutagenesis (Foster, 1999; Foster, 2000; Rosenberg, 2001). In particular, TSM refers to elevated mutagenesis observed in *E. coli* cells in which mistranslation has been increased as a result of mutations in tRNA genes or by exposure to streptomycin. TSM does not require the induction of *lexA*/*recA*-regulated SOS genes (at variance with stationary-phase mutagenesis), is suppressed in cells defective for RecABC/RuvABC-dependent homologous recombination, and is mediated by an error-prone form of DNA pol III* (Balashov & Humayun, 2002; Mamun et al., 2002).

Understanding the relationship between these pathways and the meningococcal RecB-dependent mutagenic pathway in *mutL*$^{ET\text{-}24}$ genetic background deserves future investigation.

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